Innovative Vectors for Non-viral Gene Therapy using Detergent Dialysis Technique

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DEDICATION To my Family

Content

Abstract and Scope of The Work

As a consequence of several draw backs encountered by viral technology in achieving efficient and safe gene therapy in clinical trials, non-viral gene delivery vectors are considered to date as a valuable alternative and to hold promise for future therapeutic applications.

Nevertheless, the transfection efficiency mediated by these non-viral gene delivery vectors has to be improved to benefit fully from their advantages. Cationic transfection compounds/nucleic acid complexes have been the subject of intensive investigations in recent years to understand the parameters governing the efficiency of transfection. Specifically, the understanding of such mechanisms, from the formation of the complexes to their intracellular delivery, will lead to the design of better adapted non-viral vectors for gene therapy applications.

The aim of the study described in this thesis was to investigate the detergent dialysis-based approach to form lipoplexes and polyplexes which has been previously applied to prepare non-viral lipid-based vectors for delivery of nucleic acids. This was first introduced by Reimer et al., (1995) who has demonstrated that addition of mono-cationic lipids to plasmid DNA results in the formation of a hydrophobic complex in the presence of non-ionic detergent.

This technique is based on interacting the cationic lipid and pDNA in presence of the non ionic detergent N-octyl β-D-glucopyranoside (OGP). Upon extensive detergent withdrawal by dialysis, the cationic compound /pDNA assembly is formed. It is anticipated that excess detergent removal may promote DNA condensation and aggregation which could be facilitated through intramolecular acyl chain interactions. The primary driving force for association of cationic lipid with pDNA is electrostatic; nevertheless, the hydrophobic interactions and hydration forces are still also involved in the structural rearrangements to spontaneously form lipid/pDNA complexes. It is proposed that careful control of the DNA condensation reaction is crucial in obtaining a suitable lipid or polymer-based pDNA particles for efficient delivery of genes into cells *in vitro* and *in vivo.*

Among various cationic lipids, 1,2-dioleoyl-sn-glycero-3-phosphocholine, 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and cholesteryl 3*β*-N-(dimethylaminoethyl) carbamate hydrochloride (DC-cholesterol) have become standard for cationic lipid-mediated nucleic acid delivery. In addition, a polymer based non-viral delivery system, polyethylenimine (PEI) was selected since it is the most successful polymeric gene delivery system at the moment.

In Chapter 3 we focussed on the establishment of the preparation of lipid based pDNA systems which form as a consequence of a self assembly process dependent on the formation of a hydrophobic cationic lipid/pDNA complex in presence of non-ionic detergent OGP. The influence of detergent on lipoplex assembly with regard to physicochemical characteristics of the produced lipoplexes was investigated. Additionally, we estimated the pDNA release from different cationic lipid based systems prepared by detergent dialysis technique compared to lipoplexes formed by direct simple mixing. For initiation of pDNA release, different negatively charged liposomes as model membranes were used.

Furthermore, **Chapter 4**, aimed to characterize the influence of OGP on the morphological characteristics of DOTAP/pDNA lipoplexes. The morphological features of the lipoplexes prepared by dialysis technique were visualized using cryo-TEM compared to mixed lipoplexes. Furthermore, the mechanisms of pDNA release from DOTAP/pDNA lipoplexes by different negatively charged liposomes were visualized.

In **Chapter 5** we have utilized assays including: 1) octadecyl-rhodamine (R18) de-quenching assay and 2) fluorescence resonance energy transfer (FRET) assay to assess the membrane destabilization of dialysed cationic lipid/pDNA lipoplexes in terms of lipid mixing during pDNA release by negatively charged liposomes. The membrane destabilization of lipoplexes was correlated with their pDNA release.

Finally in **Chapter 6,** the formation of pDNA/25 kDa branched PEI polyplexes in the presence of the non-ionic detergent N-octyl-beta-D-glucopyranosid (OGP) at different N/P ratios followed by dialysis was investigated. The polyplexes were characterized by laser light scattering to determine the influence of OGP on the hydrodynamic diameter and the surface charge of the particles. In addition, pDNA condensation was determined using PicoGreen® nucleic dye assay. Gene expression of the polyplexes was characterized in HEK 293 cells using flow cytometry analysis.

Reference:

Reimer D. L., Zhang Y., Kong S., Wheeler J. J., Graham R. W., and Bally M. B., Formation of novel hydrophobic complexes between cationic lipids and plasmid DNA, *Biochemistry* 34 (1995) 12877-12883.

Zusammenfassung

Aufgrund diverser Probleme, die die Verwendung viraler Genvektoren hinsichtlich Effizienz und Sicherheit aufwirft, sind nicht-virale Genvektoren derzeit als wertvolle und vielversprechende Alternativen für die Entwicklung zukünftiger therapeutischer Applikationen einzustufen.

Um von den Vorteilen nicht-viraler Genvektoren in vollem Umfang profitieren zu können, ist es jedoch notwendig, deren Transfektionseffizienz zu steigern. In den letzen Jahren waren kationische Transfektionsagens-Nukleinsäure-Komplexe Gegenstand intensiver Untersuchungen, die darauf abzielten, die die Transfektionseffizienz steuernden Parameter, zu verstehen. Vor allem das grundlegende Verständnis der hierfür relevanten Mechanismen, angefangen von der Bildung der Komplexe bis hin zu deren intrazellulärem Transportweg, schafft die Basis für die Entwicklung optimal angepasster nicht-viraler Genvektoren für gentherapeutische Anwendungen.

Das Ziel der vorliegenden Arbeit war die Untersuchung eines Dialyse-basierten Verfahrens zur Herstellung von Lipoplexen und Polyplexen, das bereits eingesetzt wurde, um nicht-virale Vektoren auf Lipidbasis für den Transport von Nukleinsäuren zu formulieren. Dieses Verfahren geht zurück auf Reimer et al. (1995), der gezeigt hatte, dass die Zugabe monovalenter kationischer Lipide zu Plasmid-DNA in Gegenwart nicht-ionischer Tenside zur Bildung hydrophober Komplexe führt.

Diese Technik basiert auf der Interaktion kationischer Lipide und pDNA in Anwesenheit des nicht-ionischen Tensids *n*-Octyl-β-D-glucopyranosid (OGP). Während der Elimination des Tensids durch Dialyse bildet sich der Komplex aus kationischem Agens und pDNA. Es wird vorausgesetzt, dass das Entziehen des Tensids die Kondensation und Aggregation der DNA fördert. Möglicherweise wird dies durch die intramolekulare Interaktion der Acylketten erleichtert. Als treibende Kraft für die Assoziation von kationischen Lipiden mit DNA sind jedoch elektrostatische Kräfte anzusehen, auch wenn hydrophobe Interaktionen und Hydratationskräfte ihren Anteil zur strukturellen Neuorganisation von spontan gebildeten Lipid-pDNA-Komplexen beitragen. Es ist anzunehmen, dass die genaue Steuerung der DNA-Kondensationsreaktion ausschlaggebend für die Gewinnung geeigneter Lipid- oder Polymer-basierter DNA-Partikel zur effizienten Transfektion von Zellen i*n vitro* und *in vivo* ist.

Aus der Vielzahl der üblicherweise verwendeten kationischen Lipide, wurden 1,2-Dioleoyl-*sn*-glycero-3-phosphocholin, 1,2-Dioleoyl-3-trimethylammoniumpropan (DOTAP) und Cholesteryl-*3β*-N-(dimethylaminoethyl)carbamat-Hydrochlorid (DC-cholesterol) als die wichtigsten Standards für kationische lipidbasierte Genvektoren ausgewählt und untersucht. Zusätzlich wurden Polyethylenimin(PEI)-basierte DNA-Partikel untersucht, da sie derzeit das erfolgreichste Genvektorsystem auf Polymerbasis darstellen.

In **Kapitel 3** wird auf die Entwicklung der Herstellungsmethodik für lipidbasierte DNA-Systeme, die sich in Folge eines Selbstassoziationsprozesses bilden eingegangen. Diese Systeme bilden sich in Anwesenheit des nichtionischen Tensids OGP in Folge eines Assoziationsprozesses, der abhängig von der Bildung des initial gebildeten Komplexes bestehend aus einem hydrophoben kationischen Lipid und DNA ist. Zur Herstellung der lipidbasierten DNA-Lipoplexe durch Dialyse wurde OGP

verwendet. Der Einfluss des nichtionischen Tensids OGP auf die Bildung der Lipoplexe, der sich in den physikochemischen Eigenschaften der Endprodukte wiederspiegelt, wurde untersucht. Zusätzlich wurde die pDNA-Freisetzung aus zwei kationischen lipidbasierten Systemen, die durch Dialysetechnik im Vergleich zur Mischungstechnik hergestellt wurden, bestimmt. Zur Aktivierung der pDNA-Freisetzung wurden die Interaktion der Lipoplexe mit negativ geladenen Liposomen als Modellmembranen genutzt.

Kapitel 4 zielt auf die Charakterisierung des Einflusses von OGP auf die morphologischen Eigenschaften von DOTAP-pDNA-Komplexen ab. Die Morphologie von Lipoplexen, die durch Dialyse im Vergleich zur Mischungstechnik gewonnen wurden, wurden anhand von cryo-TEM-Aufnahmen visualisiert. Weiterführend wurde mit dieser Technik die Interaktion von DOTAP-pDNA Lipoplexen mit verschiedenen negativ geladenen Liposomen untersucht.

Kapitel 5 fasst die Ergebnisse der Untersuchungen zur Abschätzung der Membrandestabilisierung von Lipid-pDNA-Lipoplexen während der pDNA-Freisetzung zusammen. Die Lipoplexe wurden durch Dialyse hergestellt und mittels 1) Octadecyl-Rhodamine (R18) Deuterium- Quenching und 2) Fluoreszenz Resonanz Energie Transfer (FRET) untersucht. Die Membrandestabilisierung der Komplexe wurde mit der pDNA-Freisetzung, die mittels PicoGreen®-Assay ermittelt wurde, korreliert.

Abschließend beleuchtet **Kapitel 6** die Bildung von Polyplexen aus pDNA und 25 kDA PEI (verzweigt) mit unterschiedlichen Verhältnissen von Stickstoff zu Phosphor. Die Polyplexe wurden durch Dialyse unter Zusatz des Tensids OGP hergestellt. Der Einfluss von OGP auf den hydrodynamischen Durchmesser und die Oberflächenladung der Partikel wurde mittels Photonenkorrelationsspektroskopie und der Bestimmung des Zetapotentials abgeschätzt. Zusätzlich wurde die pDNA-Kondensation mittels PicoGreen®-Assay gemessen. Die Genexpression der Polyplexe in HEK 293-Zellen wurde mittels Durchflusscytometrie bestimmt.

Referenz:

Reimer D. L., Zhang Y., Kong S., Wheeler J. J., Graham R. W., and Bally M. B., Formation of novel hydrophobic complexes between cationic lipids and plasmid DNA, *Biochemistry* 34 (1995) 12877-12883.

CHAPTER 1

Introduction

1. Introduction

1.1. Gene Therapy

1.1.1. The Principle of Gene Therapy

Gene therapy provides a unique approach to medicine as it can be adapted towards the treatment of both inherited and acquired diseases such as cancer, cardiovascular diseases, AIDS, neurodegenerative (Lasic 1997) and recently urological diseases (Goins et al., 2009). Gene delivery relies upon the encapsulation of a gene of interest, which is then ideally delivered to target cells. After uptake by endocytosis, the DNA has to be released into the cell so that transcription and translation may occur to produce the protein of interest. To achieve successful gene delivery, several barriers must be overcome at each step of this process in order to optimize gene activity (Figure 1.1).

Although the objectives and principles of gene therapy have been well-defined over the last decades, its application as a versatile, therapeutically successful approach has not yet met expectations.

Today it is clear that gene therapy not only may lead to its primary goal of replacing a deficient gene, but it could also lead to a modulation of the expression of genes acting on the physiology of malignant cells. Furthermore, by means of gene therapy, functions might be integrated into cells that are not originally present and that could serve a therapeutic purpose. Thus in a modern concept and broader sense, gene therapy refers to the potential use of nucleic acids, irrespective of whether it concerns e.g. plasmid DNA, antisense oligonucleotides or siRNA, to modulate in any kind of way the expression of genes in cells for therapeutic purposes (Cornford et al., 2009).

The number of clinical trials in gene therapy is steadily increasing, exceeding 1405 by March 2009 (data Wiley 2009, www.wiley.co.uk/genmed). “Naked” plasmid DNA is unstable under in vivo conditions due to rapid degradation by serum nucleases. Therefore, the development of gene delivery vectors play a predominate role in gene therapy. Carriers or “vectors” are necessary to provide effective DNA condensation and to protect DNA or RNA from degradation, to facilitate uptake into specific cells and finally to release DNA and to transfer the DNA or RNA into the nucleus or cytoplasm, respectively (Luo and Saltzman, 2000; Lechardeur et al., 2005).

The primary challenge for gene therapy is to develop a method that delivers a therapeutic gene (transgene) efficient to target cells where gene expression can be achieved. Depending on the vectors used for nucleic acid transfer, gene delivery is divided into two main categories: viral and non-viral gene delivery.

An ideal gene delivery method needs to meet three major criteria: (i) it should protect the transgene against degradation by nucleases in the blood circulation and intercellular matrices, (ii) it should transfer the transgene across the plasma membrane and into the nucleus of target cells, and (iii) it should have no detrimental effects.

1.1.2. Viral Gene Delivery Carriers

Viruses are the most efficient gene vectors known today due to their inherent capacity to carry and protect foreign genes, cross the cellular membrane, escape endosomes, and to achieve efficient gene expression (Walther and Stein, 2000)..

Due to their natural ability to infect cells efficiently in terms of the number of transfected cells, several viruses, such as retrovirus, adenovirus, adeno-associated virus and herpes virus, have been investigated for *in-vivo* viral-mediated gene. For example, retroviral vectors can introduce genes permanently into somatic cells by integration into cell's chromosomal DNA. Retroviruses only infect replicating cells, though the resultant permanent integration of therapeutic genes minimizes the ability to modify or to terminate therapy in response to any adverse side-effects or cure of the disease. In addition, the permanent integration of genes into host chromosomes may result in activation of oncogenes or inactivation of tumor suppressor genes.

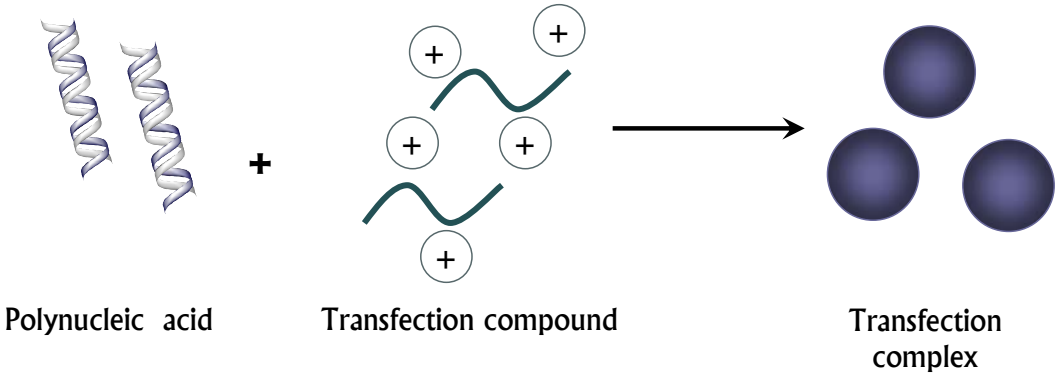
In contrast, adenoviruses efficiently infect non-dividing cells and do not integrate genes into the host genome (Mulligan, 1993; Tomlinson and Rolland, 1996; Young et al., 2006).

Different mechanisms were described for interactions of viruses with cells depending on the type of virus. The protein capsid of a virus is able to bind to proteins in the cellular membrane, gain entry as the cell internalizes, and recycles its membrane proteins. Other viruses have a protein-lipid capsid that can fuse and pass through the cell membrane (Subramanian et al., 2002). Viruses have inherent mechanisms to avoid lysosomal trafficking by promoting the fusion of the viral envelope with the endosomal membrane and therefore, causing the release of the virus into the cytoplasm (Akinc et al., 2004). These unique abilities of viruses led to the first clinical trial in gene therapy in 1990, where retroviral vectors were used to introduce the adenosine deaminase gene into the white blood cells of patients suffering from severe combined immunodeficiency (SCID) (Tomlinson and Rolland, 1996; Haider et al., 2005).

Viral vectors are able to mediate gene transfer with high efficiency up to 90 % and the possibility of long term as well as stable gene expression, and satisfy 2 out of 3 criteria described above. However, acute immune response, immunogenicity, and insertion mutagenesis especially after repeated applications in clinical trials have raised serious safety concerns about some commonly used viral vectors.

1.1.3. Non-viral Gene Delivery Carriers

The aforementioned drawbacks of viral vectors have impeded the progress of gene therapy. Avoiding these drawbacks with the use of non-viral vectors can lead to major advances in gene therapeutics. Methods of non-viral gene delivery have also been explored using i) physical (carrier-free gene delivery) and ii) chemical approaches (synthetic vector-based gene delivery).



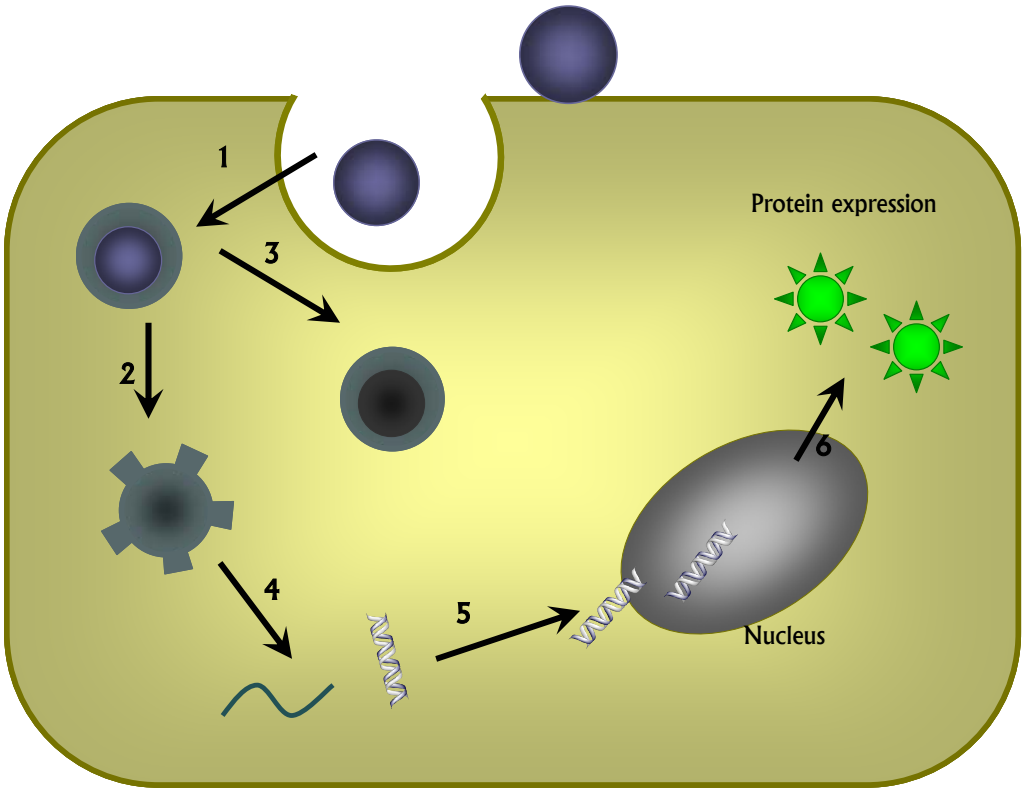


Figure 1.1: Obstacles in Gene Delivery Following *In-Vitro* Administration. The carrier system, here a transfection compound, should efficiently condense pDNA into complexes. Complexes have to attach to the cell surface, be internalized (e.g. by endocytosis) (1), escape from endolysosomes (2), move through the cytoplasm to the nucleus and cross the nuclear membrane (5) and be transcribed (gene expression) (6). In addition, the complex must dissociate and pDNA must be released (4). Alternative pathways exist for several of these steps, such as endolysosomal complex degradation (3).

1.1.3.1. Physical Gene Delivery Systems

Physical approaches include needle injection (Wolff et al., 1990), electroporation (Neumann et al., 1982; Heller et al., 2005), gene gun (Yang et al., 1990; Yang and Sun, 1995), ultrasound (Lawrie et al., 2000) and hydrodynamic delivery (Liu et al., 1999; Zhang et al., 1999) employing a physical force that permeates the cell membrane and facilitates intracellular gene transfer. Since the present work is focussed on the delivery of chemical gene delivery systems, the physical approaches will not be discussed more detailed.

1.1.3.2. Chemical Gene Delivery Carriers

Cationic lipids and polymers can electrostatically bind DNA or RNA, condense the genetic material into particles of a few tens to several hundred nanometres in diameter, protect the genes against degradation and mediate cellular entry. Such complexes of plasmid DNA with cationic lipids and polymers are known as lipoplexes or polyplexes, respectively. As opposed to the viral vectors, these systems are collectively known as non-viral gene delivery systems.

Although being less efficient, particularly in vivo (Dass, 2004), both lipoplexes and polyplexes are thought to be immunologically inert, and potentially safer than viral vectors for in vivo use. Since they are also easy to produce and to modify chemically for improvement of transfection efficiency, research efforts in this particular area have drastically increased in recent years (Li and Huang, 2000; Wang et al., 2006). Thus, numerous cationic carriers have been synthesized and for rational development structurally modified in a systematic manner in order to correlate structure with transfection activity, *i.e.* the identification of the molecular features associated with high transfection efficiency and low cytotoxicity. For example, several possibilities to reduce the toxicity of cationic amphiphiles have been discovered, among which the use of a biodegradable ester linkage between the headgroup and the alkyl tails (Aberle et al., 1998: Pijper et al., 2003).

The chemical approaches (Huang et al., 1999; Mahato and Kim, 2002; Liu et al., 2003; Neu et al., 2005) use synthetic or naturally occurring compounds such as chitosan (Masotti et al., 2008) and protamine sulphate (Biegeleisen, 2006) as carriers to deliver the transgene into cells. Although significant progress has been made in the basic science and applications of various non-viral gene delivery systems, the majority of non-viral approaches are still less efficient than viral vectors, especially for in vivo gene delivery.

Non-viral gene delivery systems provide opportunities for improved safety, greater flexibility and easier manufacturing. In contrast to viral particles, they are not limited to the delivery of coding nucleic acids but can accommodate a greater variety of cargo of different sizes, including antisense ODNs, ribozymes, siRNAs, or genes. Finally, non-viral vectors are easier and safer to produce and better amenable to chemical modifications for the purpose of effectuating therapeutic applications.

However the major drawback of non-viral vectors is their low transfection efficiency compared to viral systems which especially hinders their use in vivo therapeutic applications. A better knowledge

of the mechanism of transfection mediated by non-viral vectors in correlation to their structure will allow the development of more efficient non-viral vectors for gene therapy applications.

1.2. Molecular Shape and Aggregation Behaviour of Amphiphilic Compounds: The Packing Parameter

Cationic lipids are amphiphilic molecules, implying that they consist of a hydrophilic and a hydrophobic region, i.e. a charged cationic amine headgroup attached via a linker, for example glycerol, to a usually double hydrocarbon chain or a cholesterol derivative.

An important property of the amphiphile, with regard to its application as a vector, is its geometry. Like any amphiphile, when suspended in an aqueous environment cationic lipids can adopt various structural phases, including the micellar, lamellar, cubic and inverted hexagonal phase. The type of structure can be predicted by a factor known as the packing parameter, P. This packing parameter, $P = V/ a_0 * lc$, is defined as the ratio of the hydrocarbon volume, v, and the product of the effective head group area, a_0, and the critical length of the lipid tail, *lc* (Crystal, 1995; Hsu et al., 2005). In short, the correlation emphasizes the relevance of the ratio of the area occupied by the hydrophobic region versus that of the hydrophilic region.

The number of charges on the head group determines whether the cationic lipid will be monovalent or multivalent. In monovalent lipids, the head group consists of either tertiary or quaternary ammonium groups.

The positively charged cholesterol derivative DC-cholesterol (Fig. 1.2) was initially designed for DNA transfection (Gao and Huang, 1991) and had been used in human gene therapy trials with a good safety profile (Stewart et al., 1992; San et al., 1993; Nabel et al., 1993; Middleton et al., 1994). When used as a transfection compound, DC-cholesterol is usually combined with the helper lipid DOPE that facilitates membrane fusions and intracytoplasmic delivery of the DNA (Gao and Huang, 1991; Li et al., 1996).

It has been shown that DC-cholesterol molecules self-assemble into wormlike micelles in aqueous media (Wu et al., 2004). For gene therapy application, this lipid was usually mixed with a zwitterionic lipid to form liposomes prior to DNA complexation (Gao and Huang, 1991; Chesnoy and Huang, 2000). Despite the absence of bilayer entities for DC-cholesterol, the subsequent complexation with DNA induces formation and ordering of bilayers, where the DC-cholesterol micelles transform into a multilamellar structure after the complexation. This transformation is due to the effective dehydration of the DC-cholesterol head groups induced by the electrostatic interaction with DNA. The high packing density of DNA stiffened the membrane, thereby resulting in a topological structure of flat lamellae. When the lipid is in excess, the hydrophilic layers excluding the lipid head groups contained domains of closely packed DNA and water domains. The interhelical distance between the DNA chains confined between the fully cationic bilayers and the topological structures of the multilamellar phase were observed by transmission electron microscopy (Wu et al., 2004).

The cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) (Figure 1.2) consists of two unsaturated diacyl side chains (oleoyl), ester linker and trimethylammonium moiety in the polar head group (Lasic, 1997). Smyth Templeton et al (1997) and Liu et al (1997) reported good protection and effective delivery of DNA mixed with DOTAP: cholesterol liposome to tissue, after systemic administration into mice. DOTAP was developed through taking into account that had biodegradable ester bonds which showed lower toxicity compared to the activities of 1,2- dioleyloxypropyl-3-trimethyl ammonium bromide (DOTMA). In the meantime, Liu et al (1997) concluded that stable ether bonds may be more beneficial than the less stable ester bonds.

However, some multivalent lipids, such as lipopolyamines, have intrinsic transfection activity, and a helper lipid does not have a major impact on overall transfection activity, indicating that multivalent cationic lipids work on a different mechanism (Behr et al., 1989; El Ouahabi et al., 1997). Often, these cationic lipopolyamines have protonable amine groups that apparently intercept the endosome maturation by absorbing protons to slow down the acidification process inside the endosomes, preventing the endosome-lysosome transition.

Double-chain hydrocarbons represent the majority of cationic lipids synthesized so far. Oleoyl chain (C18:1) is the most frequently used unsaturated acyl chain, whereas C14, C16, and C18 are the commonly used saturated hydrocarbon chains. The importance of the alkyl chain length in transfection activity in vitro was studied by Felgner et al (1994), who varied the alkyl chain length in a homologous series of hydroxyethyl quaternary ammonium derivatives. They observed that the alkyl chain length can influence the transfection activity (C14:0 > C18:1 > C16:0 > C18:0). Usually, double-chain hydrocarbons are capable of forming liposomes by themselves, but they are often used in combination with a helper phospholipid in cationic lipid transfection formulations. Derivatives containing non-degradable ether bonds were found to be more toxic than those containing biodegradable linker bonds such as ester, amide, and carbamoyl bonds (Farhood et al., 1995).

1.3. Polyethylenimine as a Non viral Vector System

Among cationic polymers, PEI emerged as a very interesting candidate (Bossif et al., 1995) reaching transfection efficiencies similar to viral vectors (Abdallah et al., 1996) (Figure 1.2). The recent years have witnessed rapid development of non-viral vectors based on PEI and derivatives which possess properties addressing delivery problems associated with gene therapy.

The most prominent feature of PEI is its high cationic charge density. Condensation of the anionic DNA with the cationic PEI based on electrostatic interactions leads to the formation of compact particles of approximately 100 nm in diameter depending upon the PEI modification (Kircheis et al., 2001). Every third atom in the polymer polyethylenimine is a nitrogen atom in an amino group that can be protonated, which results in a high positive charge (Park et al., 2000; Choosakoonkriang et al., 2003). Since PEI does not contain quarternary amines, cationic charges are generated by protonation of the amine groups by the biological environment, thus leading to a correlation between

environmental pH and cationic charge density. PEI showed a level of protonation at pH 7.4 of 20 % compared to about 45 % for a pH at 5 (Suh et al., 1994; Neu et al., 2005). The wide range of apparent pKa values leads to a system with effective buffer capacity.

Polyethlyenimines (PEI) have been suggested to trigger endosomal escape of polyplexes according to the "proton sponge hypothesis". The inherent ability of PEI to facilitate DNA release into the cell is a major advantage, since release into the cytoplasm is one of the main crucial processes encountered in gene delivery. Release of transfection complexes from endosomes is critical, and delayed release of nucleic acids into the cytosol appears to result in substantial degradation (Wattiaux et al., 2000). One hypothesis, first reported by Behr in 1997, was called the "proton sponge" hypothesis. This theory postulates that a DNA delivery vector that buffers the endosomal or lysosomal compartments can propagate the rupture of these vesicles by creating an osmotic gradient between the vesicle interior and the cytoplasm.

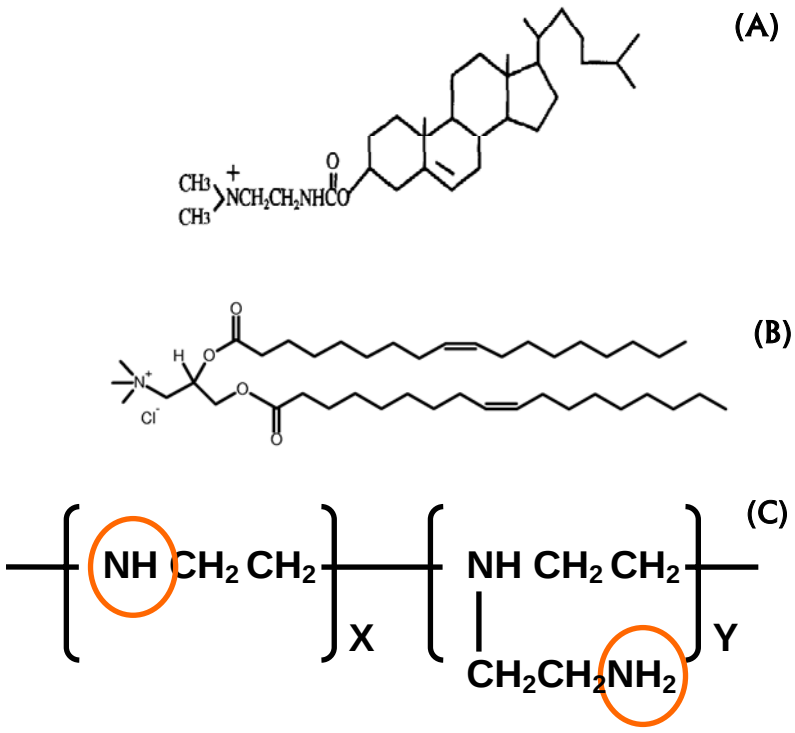


Figure 1.2: Chemical Structures of some Cationic Transfection Compounds. DC-Cholesterol (A), DOTAP (B) and branched polyethylenimine (C). DC- Cholesterol is typified by a tertiary amine head group attached to cholesterol as hydrophobic tail. DOTAP contains a quaternary ammonium head group, a glycerol linker, and two oleoyl chains as hydrocarbon tail. Polyethylenimine (PEI) shows protonatable amino nitrogens indicated by ovals.

Upon a decrease of the endosomal pH value from 7 to about 5 the charge of the PEI increases, invoking a corresponding influx of protons, negatively charged Cl^- ions and water to maintain overall electroneutrality (Fig. 1.3). Both influx ions and swelling induce an increased osmotic pressure and a destabilization of the endosomal compartment which allows release of the pDNA into the cytoplasm (Haensler and Szoka, 1993; Boussif et al., 1995; Kircheis et al., 2001; Forrest and Pack, 2002; Bieber et al., 2002; Akinc et al., 2004). In addition to direct membrane interaction, the release of polyplexes may also be attributed to the extension of the polymer network as a result of the increasing electrostatic repulsion of charged groups during acidification (Merdan et al., 2002).

Since the process of condensation is entropically driven (Bloomfield, 1997), and polyplexes form spontaneously upon mixing of cationic polymers with plasmid DNA, the degree of DNA condensation, and thus the net charge of the resulting particle, is strongly dependent on the cation to anion ratio, or more precisely the PEI nitrogen to DNA phosphate ratio (N/P ratio). Complete condensation of DNA, which means fully exploit cation/anion interactions, seems to occur at N/P ratios of 3-5 depending upon the PEI modification (Kleemann et al., 2004). However, a maximum of DNA condensation is obtained only with a high PEI extend at N/P ratios > 6, resulting in small polyplexes that exhibit a strong positive net charge.

Choosakoonkriang et al (2003) reported an optimal transfection at N/P 6-10, where small ca. 100 nm, positively charged polyplexes were formed. At N/P 6-10 it was found that 86 % of the PEI molecules were in a free un-complexed form. The DNA density inside the polyplex was found to be smaller than that in toroidal condensates induced by e.g. multivalent ions (Clamme et al., 2003). That means, only 10 % of the polyplex volume is occupied by DNA in contrast to 72 % within toroidal aggregates formed by multivalent cations (Bloomfield 1997). The net cationic charge induces the polyplexes to bind to the negatively charged glycosaminoglycans, which are present on cell membranes, and leads to the subsequent polyplex internalization by endocytosis (Ruponen et al., 2003).

PEI exists as a branched polymer (BPEI), commercially available in a broad range of molecular weights (MWs), as well as in its linear form (LPEI). The transfection efficiency and cytotoxicity (Moghimi et al., 2005) of PEI-based transfection systems depends on the MW, the degree of branching, the cationic charge density and buffer capacity of the polymer (Godbey et al., 1999b; von Harpe et al., 2000; Kunath et al., 2003). High MW BPEI has been shown to have a superior transfection efficiency compared to BPEIs with lower MW (Godbey et al., 1999b), but unfortunately, the higher transfection efficiency was accompanied by a decrease in the cell viability. Therefore, among BPEIs, a MW of 25 kDa is commonly believed to be most suitable for gene transfer. Polyplexes containing LPEIs have recently been shown to have improved transfection efficiency and cell viability compared to BPEI based transfection systems (Ferrari et al., 1997; Goula et al., 1998; Bragonzi et al., 1999; Goula et al., 2000).

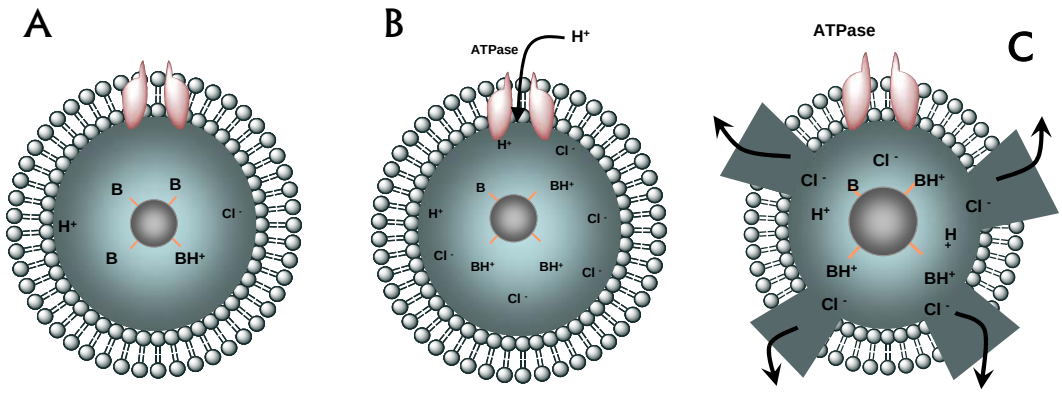


Figure 1.3: Scheme of the proton-sponge mechanism of polyethylenimine according to Sonawane et al., 2003. Polyethylenimine (PEI), which contains a large number of primary, secondary and tertiary amines, exhibit p*K*a values between physiological and lysosomal pH (A). Endolysosomes are acidified by the action of ATPases that actively transports protons from the cytosol into the vesicle. PEI polymer, therefore, undergoes large changes in protonation during endocytic process (B). It has been proposed that proton-sponge polymers prevent acidification of endocytic vesicles, causing the ATPase to transport more protons to reach the desired pH. The accumulation of protons into vesicle must be balanced by an influx of counter ions (Cl^-) and water into endocytic vesicles. The increased ion concentration ultimately causes osmotic swelling and rupture of the endosome membrane, which releases the polyplexes into the cytocol (C).

1.4. Lipoplex Formation

1.4.1. The Simple Mixing Approach

Lipoplexes are self assembling structures and their formation entails a multistep mechanism as revealed by detailed studies employing atomic force microscopy and cryo electron microscopy (Huebner et al., 1999; Oberle et al., 2000; Kennedy et al., 2000). In milliseconds electrostatic interactions between DNA phosphate and the positively charged amine head group of the cationic lipid occur, the one-sided DNA/liposome surface interaction presumably gives rise to packing constraints in the bilayer. The defects triggering extensive interactions between adjacent bilayers as reflected by extensive lipid mixing (Oberle et al., 2000; Kennedy et al., 2000; Caracciolo et al., 2005) and concomitant release of vesicle contents indicates the rupture of the membrane structure (Huebner et al., 1999; Oberle et al., 2000; Kennedy et al., 2000; Simberg et al., 2004). Rupture presumably causes exposure of hydrophobic edges of cationic membranes that may serve as nucleation sites for interaction with similar adjacent structures leading to further membrane merging, lipid mixing and aggregate growth. Compared to addition of DNA to vesicles, the reverse slows down the complex assembly process, indicating that DNA mediated vesicle-vesicle interaction is influential in destabilization rather than DNA-vesicle interaction per se (Oberle et al., 2000; Kennedy et al., 2000; Caracciolo et al., 2006). It was suggested, the cationic lipids are wrapped entirely around the plasmids.

The surfaces of the assembled complex show a smooth appearance implying proper DNA packaging. At that stage, the tendency of adjacent complexes to further undergo extensive lipid mixing decreases (Huebner et al., 1999; Oberle et al., 2000), since potential interaction sites are blocked.

Obviously, effective DNA compaction is important. In fact, the amphiphiles that are easily hydrated, form fluid aggregates, and undergo a transition to the inverted hexagonal phase in the presence of plasmid DNA at physiological ionic strength are often more favourable for effective transfection. In this so called simple direct mixing approach, kinetic control is achieved by adjusting the concentration of DNA, charge ratio, ionic strength of the solution and rate of mixing of the cationic lipid and nucleic acid. This approach relies on kinetic control to permit small particles that consist of cationic lipids and nucleic acid.

1.4.2. The Detergent Substitution Approach

In more sophisticated methods, cationic lipid and nucleic acid are inhibited from aggregation using a detergent to disrupt the bilayer structure. The addition of detergents to a bilayer forming lipid induces the formation of a number of intermediate mixed vesicles (MVs) and various types of mixed micelles (MMs) depending on detergent/lipid ratio. Afterwards, the detergent is removed by dialysis or filtration. As the concentration of the detergent/solvent decreases, the self-assembly of the lipids with the nucleic acid into lipoplexes takes place (Reimer et al., 1995; Zhang et al., 1997).

This approach was initially used for preparing stable cationic lipid/DNA particles by dissolving DNA and the cationic lipid mixture in a detergent solution followed by detergent removal (Wang and Huang, 1987; Hofland et al., 1996). The resulting particles were stable for a longer period and were more active in the presence of serum containing medium than lipoplexes prepared by the simple mixing technique (Guo et al., 2000; Xu et al., 2001). However, this method did not improve the *in-vivo* performance. Nevertheless, this approach showed that (i) a detergent solution can be used as a medium for the particle formulation because both DNA and lipids are soluble and (ii) N-octyl β-D-glucopyranoside (OGP) is a good choice as detergent since it has high critical micelle concentration (CMC) and is a non-ionic detergent which would not interfere with the charges of either DNA or cationic lipid.

The incubation of plasmid DNA with cationic lipids in presence of a detergent could result in a hydrophobic particle which is soluble in organic solvents (Reimer et al., 1995). The authors suggested the possibility that such a hydrophobic particle could be surrounded by an outer coating of lipid, which would then result in small plasmid containing particles stabilized in an aqueous medium. When the cationic lipid was added to plasmid in distilled water, the formation of large (> 1000 nm diameter) precipitates was observed. However, the subsequent addition of OGP (200 mM) resulted in solubilization of the precipitate, forming an optically clear suspension consistent with entrapment of hydrophobic plasmid DNA/cationic lipid particles within detergent micelles. However, when dialysis

was attempted to facilitate removal of the detergent, lipid based DNA particles were formed (Reimer et al., 1995; Zhang et al., 1997).

Wheeler et al. (1999) established "stabilized plasmid-lipid particles" (SPLP), produced by detergent dialysis employing a POPC/DODAC/PEG-CerC20 lipid mixture. The features of SPLP are the extended circulation lifetime of systemically administered gene therapy vectors and their accumulation at a distal tumor site. A detailed protocol for producing SPLP is provided in Phillips (2002).

1.5. DNA Condensation

DNA condensation is the collapse of extended DNA chains into compact in presence of multivalent cations, orderly particles containing only one or a few molecules of DNA. The decrease in size of the DNA domain is striking, as is the characteristic toroidal morphology of the condensed particle, so the phenomenon of DNA condensation has drawn considerable attention.

When a DNA molecule is tightly packed, almost all of the negative charges should be neutralized, accompanied by the enhancement of counter-ion condensation release. More precisely, the volume part of the compact DNA is fully neutralized whereas negative charge remains on the surface (Yamasaki et al., 2001). It is obvious that an abrupt change in the degree of association of counter-ions is caused by the discrete transition between the elongated coil and folded compact states. In other words, the charge neutralization by cationic species has a significant contribution to the free energy change of the folding transition.

There are essentially two challenges for the dominant attractive force in condensation: hydration forces and correlated counter-ion fluctuations (Bloomfield, 1997). Condensing agents generally act either by decreasing repulsions between DNA segments e.g. neutralizing of phosphate charge, reorienting water dipoles near DNA surfaces, by multivalent cations, or by making DNA-solvent interactions less favourable, for example by adding ethanol, which is a poorer solvent than water for DNA, or by adding another polymer, such as polyethylene glycol (PEG), which excludes volume to the DNA.

When the alcohol concentration increases, discrete toroids and rods are replaced by more extensively aggregated structures. With sufficient alcohol able to reduce the dielectric constant below 65 as well as in the presence of Co $(NH_3)_6^{3+}$, DNA collapses into a network of multistranded fibers (Arscott et al., 1995). Using circular dichroism spectroscopy, a study indicates that the DNA has undergone a B-A transition, although at concentrations at which neither ethanol nor Co $(NH_3)_6^{3+}$ could induce the transition alone. The A-DNA then apparently strongly self-adheres and rapidly aggregates into fibrous networks, not allowing more compact and ordered condensates to form. Multivalent cations may also cause localized bending or distortion of the DNA, which can also facilitate condensation.

DNA condensation is a readily reversible process, which depends on the association of sufficient condensing compound with the DNA (Bloomfield, 1997). DNA condensation by hexaamine cobalt (III) (Widom and Baldwin, 1980; Conwell and Hud, 2004) has been studied using light scattering kinetic of in dilute DNA solutions (1–5 μg/ml). These experiments typically show an initial rapid increase in scattering intensity over the first few minutes followed by a slower increase to a plateau after 30-120 minutes. The increase in intensity is attributed to a collapse of individual or association of several DNA molecules resulting in a larger particle structures. Further moderate increase in intensity after several hours may be due to secondary aggregation of toroids or rods.

Much attention has been paid recently to the condensation of DNA with cationic liposomes, since the lipoplex can be an efficient agent for transfection of eukaryotic cells. This is presumably because the condensed state of the DNA protects it from nucleases (Van der Woude et al., 1995; Xu and Szoka, 1996) and allows better binding to the negatively charged cellular membrane and hence lipid coating increases the permeability of DNA through cell membranes.

1.6. Morphology of Cationic Lipid/pDNA Assembly

Addition of DNA to the lipid dispersion leads to a spontaneous formation of discrete complexes. The structures formed between cationic vesicles and DNA are called lipoplexes. Such structures have attracted large interest due to their potential application in gene therapy (Audouy and Hoekstra, 2001; Dass, 2002; Simberg et. al., 2004). DNA condensation and lipid restructuring are known to occur during lipoplex formation. Lipid restructuring involves both liposome fusion (Gershon et al., 1993; Mok and Cullis, 1997) and release of the vesicles' aqueous content (Kikuchi and Carmona-Ribeiro, 2000; Kennedy et al., 2000).

For characterization of parameters such as the effect of homogeneity in size and morphology, microscopic techniques of sufficient resolution were highly useful, because they may provide further insight into the mechanism of lipoplex assembly (Simberg et al., 2001). Freeze–fracture electron micrographs of DNA mixed with cationic liposomes led to the hypothesis that intact liposomes bind to DNA at low concentration and fuse to encapsulate the DNA at higher concentrations (Sternberg et al., 1994). The authors postulated that during the cationic lipid/DNA assembly at low DNA/lipid ratios aggregated globular lipid structures known as "meatballs" covered by DNA tubules known as "spaghetti" like structures were formed, DNA tubules were found to be connected to the liposome complexes as well as occurred free in suspension (Fig. 1.4). Cyro-TEM studies showed further evidence for liposome restructuring like elongated rod-like structures (Gershon et al., 1993) and aggregates of globular particles (Sternberg et al., 1994; Mok and Cullis, 1997; Eastman et al., 1997).

Gershon et al (1993) have proposed another model for cationic lipid/DNA complexation. At low ratios of liposomes to DNA, positively charged lipid vesicles are adsorbed to nucleic acids to form aggregates that gradually surround larger segments of the DNA. When the amount of liposomes is increased, the aggregated liposomes along the DNA reach critical concentrations and charge densities

at which membrane fusion and cooperative DNA collapse processes are initiated. After increase of the liposome concentration, the collapsed DNA structures are covered completely by the lipid bilayers. The size of the final product is dependent on the size of the initial lipid vesicles (Goncxalves et al., 2004), the positive-to negative charge ratio and the ionic strength of the medium. The proposed appearance of the complexes showed clusters of aggregated dense particles.

Cryoelectron microscopy (Gustafsson et al., 1995; Lasic et al., 1997; Huebner et al., 1999; Schmutz et al., 1999) and small angle x-ray scattering studies of such aggregates (Lasic et al., 1997; Rädler et al., 1997; Boukhnikachvili et al., 1997) reveal an internal multilamellar structure where lipid bilayers alternate with hydrated DNA monolayers. This lamellar structure coexists with an inverted hexagonal structure when the cationic liposomes contain dioleoylphosphatidylethanolamine (DOPE) at molar ratios greater than 0.41 (Koltover et al., 1998; Lin et al., 2000).

A similar study was performed by Gustafsson et al (1995) using cryo transmission electron microscopy of cationic liposomes composed of DOTAP or dimethyl dioctadecylammonium bromide (DDAB) and DOPE mixed with plasmid DNA revealed entrapment of DNA into aggregated multilamellar structures at low lipid-DNA ratios. When the amount of DNA was increased, plasmid DNA was found to be associated on the surface of the complexes.

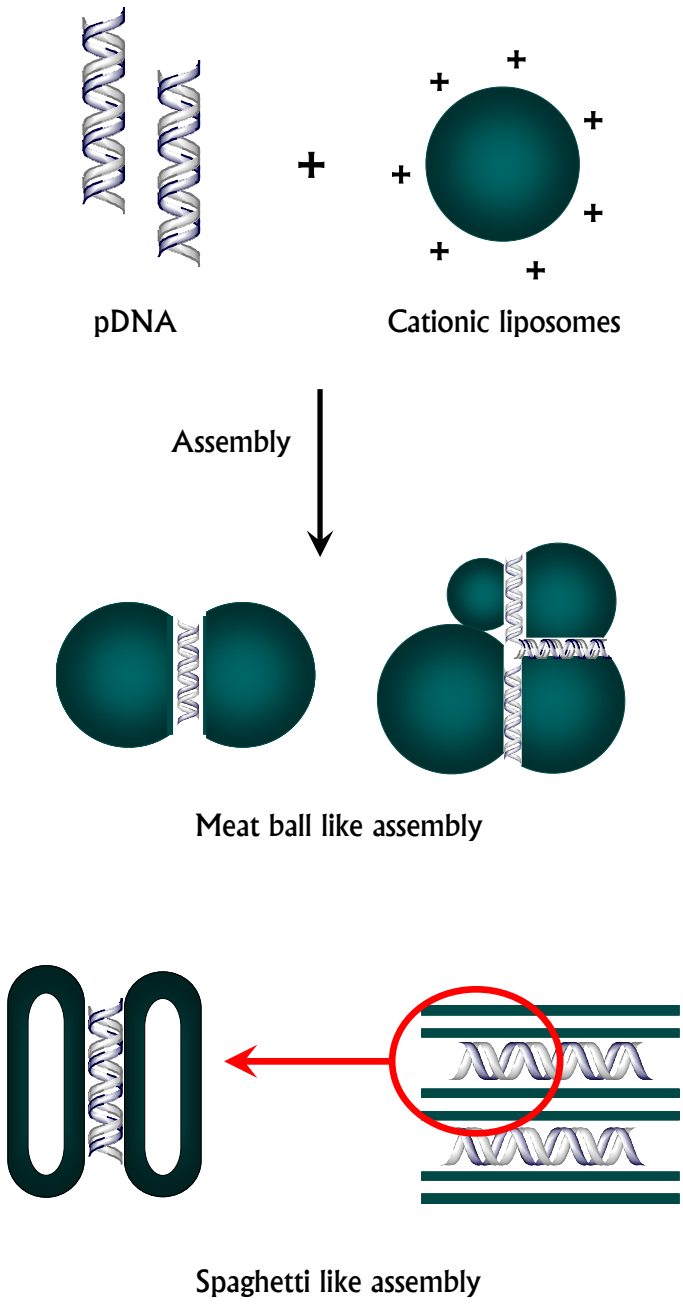


Figure 1.4: Scheme showing lipoplex assembly as proposed by Sternberg et al., (1994), based on freeze-fracture electron micrographs. Negatively charged DNA with the cationic DC-cholesterol liposomes formed cationic lipid/DNA aggregates without spaghetti-like structures "meat ball like aggregates" occurring at short incubation times and low DNA/lipid ratios, and spaghetti-like structures, at longer incubation times and higher DNA/lipid ratios.

1.7. Gene Delivery Associated Barriers

The cationic compounds interact spontaneously with the negatively charged DNA. The mechanism of non-viral gene transfer is thought to follow an endocytotic process. In order to lead to successfully high transgene expression, various biological barriers must be crossed: (i) interaction with the plasma membrane and uptake by endosomes, (ii) escape from the endosome into the cytoplasm, (iii) trafficking from the cytoplasm into the nucleus and (iv) nucleus entry (Fig. 1.1).

1.7.1. Cellular Uptake and Intracellular Distribution of DNA complexes

Both, cationic polymers and lipids have shown promise as gene delivery agents since their polycationic nature produces particles that pass one or more of these barriers. For example, by collapsing DNA into particles of reduced negative or increased positive charge binding to the cell surface by electrostatic interaction and enhanced endocytosis may be promoted (Boussif et al., 1995; Haensler and Szoka, 1993). In many cases, cationic polymers seem to produce more stable complexes, thus offering more protection during cellular trafficking than cationic lipids (Zabner et al., 1995; Pollard et al., 1998; Hwang and Davis, 2001).

DNA by itself associates very poorly with the negatively charged cell membrane because of charge repulsion. Positively charged complexes interact by electrostatic interaction with negatively charged cell surface domains. Therefore, a high positive zeta potential of transfection complexes is an important step to accomplish cellular interaction and uptake. Complexes composed of polycations with molecular weights of several thousands exhibit comparably higher zeta potentials (Ruponen et al., 1999; Jeong et al., 2001; Ahn et al., 2002) irrespective of the polycations used.

Internalization events seem to be affected not only by charge, but also by the size of complexes used for transfection. Given the heterogeneity of cationic compound/DNA complexes, it has been difficult to define precisely the effect of complex size on transfection and at what levels in the overall transfection process size might be relevant. In recent study, Rejman et al (2004) have shown that particle size as such can strongly affect the efficiency of cellular uptake, the mode of endocytosis and the subsequent efficiency of particle processing along the endocytic pathway. They demonstrated that particles of about 500 nm could be internalized by non-phagocytic cells by a clathrin-independent pathway. Particles with diameters less than 200 nm, and approx. 80 % of the 200 nm particles as such, are internalized via clathrin-coated pits. The upper limit is about 1 μm since these particles were not internalized by the non-phagocytic B16 cells investigated in this study.

A previous study showed that complex properties of linear poly-L-lysine, intact polyamidoamine (PAMAM) dendrimers, fractured dendrimers, and branched PEI with DNA were similar in terms of size and zeta potential. Nevertheless, high levels of gene expression could only be achieved by fractured dendrimers and branched PEI presumably due to the stability of the complexes and absence of aggregation (Tang and Szoka, 1997).

1.7.2. Endosomal Escape

During the cellular process of endocytosis, early endosomal vesicles become acidified by the vacuolar-ATPase proton pump (Figure 1.3), producing a large vesicular proton gradient versus cytosolic pH (Tycko and Maxfield, 1982). The pH inside endosomal compartments could become as low as pH 5.5-4.5. It has been demonstrated that escape of DNA from the endocytic compartments is one of the major barriers to efficient gene delivery (Amiji, 2005).

A key question in terms of the trafficking of complex along the endocytic pathway is the mechanism by which material moves through the endosomal compartments. Regarding this issue two different hypothesis were proposed (Helenius et al., 1983; van Deurs et al., 1989); i) the vesicle shuttle model and ii) The maturation model.

The vesicle shuttle model proposed that early endosomes represent stable, long-lived organelles, and complex destined for the lysosomes would be packed into vesicles that bud from one endosomal membrane compartment and fuse with the next.

The maturation model predicts that the complex taken up by endocytosis is contained within an organelle composed initially of internalized plasma membrane and that with time this organelle develops into a secondary endosome and then a late endosome (Lloyd and Mason, 1996). It is generally believed that failure of DNA to escape to a significant extent from the endosomal compartment constitutes a major barrier to efficient DNA complex mediated transfection. If a non viral vector has the ability to disrupt or fuse with the endosomal membranes, the released gene can escape from the endosome into the cytosol, and effective transgene expression should occur.

Endolysosomal escape may also be exemplified by endosomolytic polycationic polymers such as PEI. Behr (1997) postultated the so-called ‘proton sponge hypothesis’, which relates the intrinsic endosomolytic activity of PEI to its capacity to buffer the endosomal environment, prompting the osmotic swelling of the vesicle and finally its rupture, which leads to the liberation of the DNA/PEI complexes (polyplexes) into the cytoplasm (for details see section 1.3). Another mechanism for endolysosomal escape of polyplexes was suggested by Bieber et al. (2002). Using electron microscopy , endosomal membrane holes have been observed and were related to the direct interaction of high molecular weight (MW) branched PEI (BPEI) (800 kDa) with the endosomal membrane in a non-acidic environment. The authors suggested that low molecular weight (MW) PEIs (25 kDa) also induce minor membrane damages, but that those holes may be quickly resealed. In addition to direct membrane interaction, the release of polyplexes may also be attributed to the extension of the polymer network as a result of the increasing electrostatic repulsion of charged groups during acidification (Merdan et al., 2002).

An alternative pathway for endosomal escape is considered to be fusion of the cationic lipid/pDNA complex with endosomal membranes (or membrane destabilization). This appears to be necessary for the effective delivery of the nucleic acids from the lipoplex to the cell (Düzgüneş, 2004). Different mechanisms have been proposed for DNA release from lipoplexes. For lipoplexes, endosomal

membrane destabilization is thought to result from the intermingling of lamellar phase perturbing lipids, including inverted hexagonal phase H_{II} preferring cationic lipids, with the endosomal membrane. Zelphati and Szoka (1996) have proposed a model that describes nucleic acids release from the endosome. After internalization of the DNA complexes by endocytosis, the endosomal membrane is destabilized, which causes a flip-flop of anionic lipids from the cytoplasmic face of the endosome into the internal face. The anionic lipids interact with the cationic lipids, displacing the nucleic acid from the cationic lipid and allowing the release of the anionic nucleic acid from the lipoplex into the cytoplasm of the cell. The presence of negatively charged lipids DOPS may amplify this destabilizing non-bilayer membrane organization upon its interaction with the cationic lipid and simultaneously cause the competitive dissociation of the DNA from the lipoplex.

Another strategy to avoid the endolysosomal pathway is the extra-cellular release and subsequent diffusion of the transfection complex over the plasma membrane. This may provide an effective way to achieve cytosolic delivery. The so-called cell-penetrating peptides (CPP) have been reported to accomplish direct cytosolic delivery when attached to various carriers including liposomes (Torchilin et al., 2001; Tseng et al., 2002). This could be achieved by co-encapsulation of fusogenic peptide, surface coupling of CPP to liposomes surface and photochemical internalization.

Co-encapsulation of fusogenic peptides such as N-terminal domain of viral protein hemagglutinin subunit HA2 induces membrane destabilization in the lysosomes. Upon acidification, this peptide domain becomes protonated, causing a conformational change from random coil to alpha helix (Skehel et al., 1982; Doms et al., 1985). Due to this conformational change, the fusion peptide is inserted into the endosomal membrane and destabilizes it (Harter et al., 1989; Stegmann et al., 1991).

Mastrobattista et al. (2002) studied the co-encapsulation of the influenza virus-derived synthetic diINF-7 whether it could enhance the cytosolic delivery of liposome entrapped proteins. They showed that the alpha helical content increased from 15% to 31% when the pH was lowered from 7.4 to 5.2. The coupling of TAT-peptide to liposomes surface also greatly enhanced the cellular binding and subsequent uptake of the liposomes (Fretz et al., 2004).

A novel photochemical technique, named photochemical internalization (PCI), was developed for inducing release of molecules from endocytic vesicles (Hogset et al., 2004). The photosensitizer can preferentially localize in endosomal membranes. Upon illumination of the photosensitizers, highly reactive oxygen species are formed and the endosomal membrane is damaged and the carriers will be released into the cytosol.

1.7. 3. Entry of DNA into the Nucleus

Entry of DNA into the nucleus after its release into the cytosol is necessary for gene expression (Chesnoy and Huang, 2000). The mechanism of the nuclear delivery of the lipid-DNA complex is believed to be an inefficient process (Fig. 1.1). The aqueous channels of the nuclear pore complex allow the free diffusion of small macromolecules (<70 kDa), but the transport of larger

macromolecules requires an active process (Dowty et al., 1995). Remy et al. (1995) formulated a virus-like particle containing lipospermine as cationic lipid and a lipid bearing a head group of a nuclear localizing signal peptide (NLS). They did not notice any improvement in transfection. Zabner et al. (1995) showed that intact lipid-DNA complexes microinjected into the cytoplasm of *Xenopus* oocytes had only low transfection activity. Thus it can be concluded that DNA has to be released from lipids to enter the nucleus. The efficiency of entry of lipoplexes into the nucleus is very low. This was observed in the case of lipoplexes, where fluorescent labelled DNA and lipids were used. Labelled DNA appeared in the nucleus whereas the cationic lipids did not. This suggests that lipoplex disassembly takes place before the DNA reaches the nucleus (Marcusson et al, 1998). However, in the case of polyplexes composed from PEI and pDNA, PEI not only accompanied the nucleic acid to the nucleus but moreover targeted into the nucleus (Boussif et al, 1995; Pollard et al, 1998; Godbey et al, 1999a; Wightman et al, 2001).

Another drawback of the nuclear transport of DNA is its instability in the cytoplasm. However, Zanta et al (1999) reported that a single nuclear-localization signal peptide linked to one end of a gene is sufficient to promote up to 1000-fold higher transfection levels compared with naked plasmid DNA lacking the single nuclear localization signal peptide.

1.8. Conclusive Remarks

Significant effort in research has been dedicated to the development of lipidic and/or polymeric nanoparticular nonviral gene carriers in the past 10 years, but there are still obstacles that have to be overcome. Likewise, since the uptake of non-viral carries occurs mainly via endocytosis, in order to exert its activity, the pDNA must be released from the endo- and lysosomes. Additionally, it must be transported to the assigned organelle. It is important to note that strong binding and efficient DNA condensation do not correlate directly with gene delivery efficiency, probably because tight binding prevents transcription. One important step involved in the process leading to transfection is the release of the DNA from the complex. Only free DNA would be able to interact with target structures in the cells and can be transcribed and translated into the protein structures.

Therefore, disassembly of the complexes must occur before mRNA transcription can proceed. Both the stability of complexation and the rate of dissociation influence transfection efficiency. In other words, the disassembly of DNA complexes diametrically opposes the previous stages of gene delivery; premature dissociation could lead to DNA degradation and low efficacy of gene delivery, while delayed or incomplete dissociation would interfere with efficient gene expression (Schaffer et al., 2000).

A transfection compound must therefore balance sufficient binding strength to initially protect the plasmid with the ability to release the plasmid, perhaps by competitive binding of genomic DNA, cytosolic proteins or anionic membrane lipids (Zelphati and Szoka; 1996; Schaffer et al., 2000).

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CHAPTER 2

Materials and Methods

2.1. Materials

PicoGreen® was obtained from Molecular Probes **(**Invitrogen**,** Eugene, OR, USA) and N-octyl β-D-glucopyranoside (OGP) was purchased from Carl-Roth GmbH, Karlsruhe, Germany. Trizma® pre-set crystals pH 7.4 (Sigma, St. Louis, MO, USA), HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (Carl-Roth GmbH, Karlsruhe, Germany) and sodium chloride (NaCl) (Fluka, Deisenhofen, Germany) were used for buffer solutions.

2.1.1 Lipids

The phospholipids 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2 dioleoyl-sn-glycero-3-phospho-l-serine (DOPS), the cationic lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 3*β*-N-(dimethylaminoethane) carbamoyl cholesterol, oleic acid and the fluorescent lipids 1,2-dioleoyl-sn-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl)-ammonium salt (NBD-PE), N-1,2-dioleoyl-sn-Glycero-3 Phosphoethanolamine-N- (lissamine Rhodamine B sulfonyl) (Rhodamine DOPE) and octadecyl rhodamine B chloride (R18) were purchased from Avanti Polar Lipids Inc., Alabaster, USA. All lipids were used without further purification as stock solutions in chloroform and were stored at –20 °C.

2.1.2. Polymers

The polyethylenimine (PEI) used in this work was Lupasol® WF (BASF, Ludwigshafen, Germany). It is a technical grade 25 kDa, branched polyethylenimine in a 50 % watery solution and was a kind gift from BASF. Before being used for complexation experiments, PEI was diluted to 0.9 mg/ml in 10 mM TRIS buffer, and pH was adjusted to 7.4. The solution was sterile filtered using Sterifix 0.2 µm filter (Braun Melsungen AG, Melsungen, Germany), aliquoted and stored at 8 ºC.

2.2. Methods

2.2.1. pDNA

The plasmid deoxyribonucleic acid (pDNA) used in this study was the plasmid EGFP-C3 (Clontech Laboratories, Mountain View, CA, USA) coding for enhanced green fluorescence protein. The plasmid was amplified in the Escherichia coli strain (E. coli) XL-Blue MRF supercompetent cells (Stratagene, La Jolla, CA, USA). This seed was used to inoculate 150 ml Luria Bertani medium (Carl-Roth GmbH, Karlsruhe, Germany) containing 30 µg of the selective aminoglycoside antibiotic kanamycin (Carl-Roth GmbH, Karlsruhe, Germany). Plasmid was extracted from E. coli cells using the GenElute Endotoxin-free Plasmid Maxiprep Kit **(**Sigma**,** Deisenhofen, Germany) following manufacturer's instruction. UV-spectroscopy showed no presence of protein contamination in all

pDNA batches: the ratio of absorbance of 260 nm and 280 nm was about 1.8–1.9. Calf thymus DNA (Fluka, Deisenhofen, Germany) was used for pDNA condensation assays.

2.2.2. Lipoplex Preparation

2.2.2.1. Mixed Lipoplexes

Lipoplexes were prepared by two strategies. In strategy I, lipoplexes were formed by conventional mixing of cationic lipid and pDNA.

The thin-film hydration method was used to prepare the cationic lipid films as described before (Wang et al., 2006). The cationic liposomes were prepared by mixing chloroform or chloroform methanol (3:1, v/v) solutions of the lipids in round bottom glasses followed by removal of the chloroform by a stream of nitrogen gas to produce dried lipid films. Residual solvent was removed by exposing the dry film to high vacuum for at least 2 h. The lipid film was re-suspended in 20 mM HEPES containing 145 mM NaCl (pH 7.4). Cationic lipoplex compositions included DOTAP and DC-cholesterol. The cationic lipid/pDNA complexes in most of the experiments were prepared by mixing equal volumes of liposomes and 0.1mg /ml pDNA at N/P ratios 4 and 5. Afterwards lipoplexes were ultra-sonicated in an ultrasonic bath (Bender Hobein, Zürich, Switzerland) for a few seconds and allowed to incubate for 30 min at room temperature before use.

2.2.2.2. Dialysed Lipoplexes

Alternatively in strategy II, lipoplexes were prepared by detergent dialysis as described previously (Wheeler et al., 1999; Hafez et al., 2000). Lipoplexes were prepared in the presence of the non-ionic detergent OGP followed by detergent dialysis. Plasmid DNA was diluted to 0.1 mg/ml pDNA in 20 mM HEPES, 145 mM NaCl buffer containing 200 mM detergent OGP at pH 7.4. This solution was used to reconstitute the cationic lipid films prepared as described in section 2.2.2.1. After 20 min incubation at room temperature, the mixture was subjected to dialysis against 2 L of the same buffer for 48 h at room temperature with changing the buffer twice. Under the chosen conditions pDNA and cationic lipids were prepared with the following cationic lipid/pDNA molar ratio, (ρ) = L_C/D = (moles of cationic lipid)/ (moles of pDNA bases) of 4 and 5. According to previous studies (Gershon et al., 1993) this would be a condition in which the majority of pDNA molecules should be complexed.

2.2.3. Polyplex Preparation

Polyplexes composed of polyethylenimine and the plasmid EGFP-C3 were prepared by diluting plasmid and the appropriate amount of polymer separately with 10 mM Tris buffer, pH 7.4, to equal volumes according to the protocol of Boussif et al., (1995).

Calculation of Nitrogen to Phosphate (N/P) Ratio

The required amounts of polymer and plasmid were calculated according to the desired N/P ratio based on previous results (Fischer et al., 1999). The N/P ratio is the ionic balance of the PEI/pDNA polyplexes. The positive charge of PEI is originated from the nitrogen (N) of the repeat unit of PEI, NHCH2CH2, which has a molecular weight of 43 g/mol. The negative charge in the plasmid DNA backbone arises from the phosphate group (P) of the deoxyribonucleotides. The average molecular weight of a nucleotide is assumed to be 330 g/mol.

Two strategies were used for preparation of the polyplexes. Mixed polyplexes of plasmid DNA and PEI were prepared by mixing of both components leading to complex formation based on electrostatic interactions. Additionally, dialysed polyplexes were performed by an alternative technique complexing pDNA and PEI in the presence of the nonionic detergent OGP followed by dialysis. Plasmid DNA was complexed with polyethylenimine at different nitrogen/phosphate (N/P) ratios.

2.2.3.1. Mixed Polyplexes

Mixed polyplexes at nitrogen/phosphate ratios 5 and 10 were formed as follows: The polymer solution was added to pDNA in 10 mM Tris buffer at pH 7.4. The mixture was sonicated in an ultrasonic bath (Bender Hobein, Zürich, Switzerland) for few seconds and allowed to incubate at room temperature for 30 min before use. All samples were prepared in triplicates.

2.2.3.2. Dialysed Polyplex

For dialysed polyplex formation, polyplexes were formed by mixing plasmid DNA and PEI in the presence of the non-ionic detergent OGP as described previously (Wheeler et al., 1999; Hafez et al., 2000). The required amount of plasmid DNA was diluted in 10 mM Tris, pH 7.4 containing 50, 100 or 200 mM nonionic detergent OGP. The solutions containing pDNA and OGP were allowed to stand at room temperature for 30 min before addition of PEI solution. After PEI addition, incubation in 10 mM Tris buffer at pH 7.4 for 30 min was completed before the plasmid DNA/PEI mixture was dialysed against 2 L 10 mM Tris, pH 7.4 for 48 h with 2 buffer changes.

2.2.4. Photon Correlation Spectroscopy

The hydrodynamic diameter of complexes was determined by photon correlation spectroscopy. Measurements were performed using a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) with laser beam of 633 nm. The scattered light was detected at 173° angle. The viscosity (0.88 mP) and the refractive index (1.33) of distilled water were used for data analysis. Thirty minutes before measurement, the pDNA complexes were diluted 1:50 in 10 mM Tris buffer, pH 7.4. Sampling time was set to 5 min, and three measurements with 4 sub-runs were performed for each sample. The

samples were thermostated to 25 °C. Data were evaluated with the Dispersion Technology Software version 5.10 (Malvern Instruments).

2.2.5. Zeta Potential Measurement

pEGFP-control plasmid was complexed by simple mixing or dialysis techniques with the appropriate amount of polymer or lipid in 1 ml water as described above. Zeta potential measurements were carried out in the standard capillary electrophoresis cell in position 2 mm using a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) at 25 °C. The measurements were performed in filtered (0.2 µm) de-ionized water. The sampling time was set to automatic. Average values were calculated with the data from 20 runs. Zeta potential was calculated according to the Smoluchowski model equation using the Dispersion Technology Software version 5.10 (Malvern Instruments).

2.2.6. pDNA Condensation of Polyplexes

pDNA condensation was examined by determining pDNA accessibility to the nucleic acid intercalating dye PicoGreen®. pDNA unpacking was measured by dequenching of PicoGreen® fluorescence at different pHs as described previously (Moret et al., 2001). Briefly, triplicates of 100 µg calf thymus DNA were complexed with polymer at nitrogen/phosphate ratios of 5 and 10 in 10 mM Tris buffer, pH 7.4. The pH was adjusted to 7.3, 8 and 11.6. Plasmid DNA was used in a concentration of 2 µg/ml for each polyplex. Fifty microlitres polyplex solution were added to each well of a 96-well plate. 50 µl PicoGreen® (1:200) were added per well. The plate was gently shaken on a plate shaker (Titramax 100, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at 125 rpm. Fluorescence was subsequently recorded at excitation wavelength λ_{ex} 485 nm and emission wavelength λ_{em} 520 nm (Jeffs et al., 2005) using a plate reader (Fluostar optima, BMG Labtechnologies, Offenburg, Germany).

Plasmid concentration in polyplexes was determined by measuring fluorescence upon addition of PicoGreen® dsDNA Quantitation probe (Molecular probe) to the polyplexes (F_i) and comparing this value to that obtained upon increasing pH to 8 or 11.6 (F_{pH}). Each well was measured three times with an average of 9 samples, and the mean was calculated. Naked pDNA as control was also processed at different pHs in the same way as indicated for the polyplexes. Results were transformed into relative fluorescence values as described in the literature (Moret et al., 2001). A value of 1 is attributed to the fluorescence of PicoGreen® with naked pDNA. A value of 0 is assigned to the residual fluorescence of PicoGreen® without pDNA.

2.2.7. Cell Culture

The transfection efficiency of pDNA delivered by PEI was examined by green fluorescence protein (GFP) expression as a gene reporter using Fluorescence Activated Cell Sorting Technique (FACS). HEK 293 (human embryonic kidney) cell line was obtained from the Institute of Cell Biology and

Immunology, University of Stuttgart as a gift. Cells were cultured in Earle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and 2 mM L-glutamine (PAA Laboratories, Linz, Austria) at 37 ºC, 5 % CO_2 and 95 % relative humidity.

2.2.8. Transfection Experiment

Transfection experiments were performed in quadruplicate. Briefly, HEK 293 cells were seeded in 12 well-plates at a density of 50,000 cells/well 24 h before transfection. Afterwards, medium was replaced by fresh MEM (400 µl) without FCS containing polyplexes of pEGFP and 25 kDa PEI (N/P 5, 10, 15 and 20) at final concentrations of 2 and 3 µg pDNA per well. After the volume was completed to 1 ml medium without FCS in each well, cells were incubated for transfection. Five hours later, the medium was discarded and replaced by the full growth medium at 37 °C in 5 % CO_2. Cells were harvested after 24 and 48 h. Cells were washed twice with Phosphate Buffered Saline (PBS), detached by gentle pipetting and checked by microscopy to ensure at least 85 % of the cells were detached. The cell suspension was then transferred to FACS polystyrene test tubes (12×75 mm, Falcon). Cell sample treated with free pDNA were used as control.

For assessment of gene expression, flow cytometric analysis was carried out by using a flow cytometer (Epics XL MCL, Beckman Coulter GmbH, D-Krefeld, Germany) equipped with an air-cooled argon laser (wavelength: 488 nm). The emitted fluorescence of green fluorescence protein was detected at the corresponding photomultiplier tube (PMT) at the FL1 channel (515–545 nm). Forward laser light scattering (FSC) is useful in estimating the size and shape of cells, while side scattering (SSC) at 90° to the laser beam indicates cell granularity or internal complexity of various cell populations. The corresponding discrimination window (gate) is set in the cytogram in which the main cell pool is selected to avoid small (debris) and big (aggregates) particles, based on the forward and side scattering data.

The fluorescence intensity is given by the X-axis of the histogram, while the number of events (number of cells) is given by the Y-axis. Ten thousand events were accumulated for every sample, and the fluorescence intensity range (M1) was set as a threshold throughout the experiments. For EGFP detection, the percentage of fluorescent cell sorting events in the established threshold (M1) was reported with correction for the background fluorescence of the control sample with λ_{ex} 495 nm and λ_{em} 518 nm. Measurements were carried out with a flow speed counting until 250 events per second. The percentage of transfected cells was counted based on the two peaks in the FL1 channel, which reflects: (1) the presence of transfected cells in the population that have synthesized green fluorescent protein (GFP) and (2) non-transfected cells with autofluorescence only.

2.2.9. Preparation of Anionic Liposomes (AL)

Liposomes containing anionic lipids were prepared as described previously (Tarahovsky et al., 2004). Lipid films were prepared from chloroform solutions containing appropriate amounts of lipids, as shown in Table 2.1. The solvent was evaporated under a stream of nitrogen to obtain a thin lipid film which was hydrated in 20 mM HEPES buffer containing 145 mM NaCl at pH 7.4 to form multilamellar vesicles (MLVs) at 10 mM lipid concentration. Unilamellar vesicles with a size of about 100 nm were then prepared by extrusion through polycarbonate membranes with different pore sizes (400 nm, 200 nm, 100 nm, 50 nm, Armatis, Mannheim, Germany) with the help of a Liposofast-Miniextruder (Avestin, Ottawa, Canada, MacDonald et al., 1991). The lipid mixture was subjected to extrusion decreasing pore sizes from 400 to 50 nm 21 times for each membrane at room temperature. Liposomes were evaluated by photon correlation spectroscopy.

Anionic liposomes	Composition	Molar ratio
I	DOPS/DOPE/DOPC	2.5:2.5:5
II	OA/DOPE/DOPC	2.5:2.5:5
III	DOPS:DOPC	3 :7
IV	OA: DOPC	3 :7
V	OA/DOPE	3 :7

Table 2.1: Composition of Negatively Charged Liposomes

2.2.10. pDNA Release

Lipoplexes were prepared at (ρ) of 4 and 5 according to the approaches described (Tarahovsky et al., 2004; Wang et al., 2006; Koynova and MacDonald, 2007) Lipoplexes were then diluted to an amount of 2 μg pDNA/ml followed by addition of PicoGreen® solution 1:200 dilution (Molecular Probe). Anionic liposomes (AL)/lipoplex mixed dispersions were prepared by mixing solutions of AL and preformed lipoplexes at different charge ratios $R=A/Lc$ (moles of anionic lipid/moles of cationic lipid). Volumes containing 4 fold more negative charges than positive charges were added to accomplish pDNA release. Fifty microlitres of each solution were added to each well of a 96-well plate. The change of fluorescence was subsequently monitored using three different kinetic windows as shown in Table 2.2, at excitation wavelength λ_{ex} 485 nm and emission wavelength λ_{em} 520 nm using a plate reader (Fluostar optima, BMG Labtechnologies, Offenburg, Germany). Data of release were calculated from the fluorescence level obtained at certain time points, corrected for the background value obtained prior to addition of anionic vesicles, relative to the fluorescence obtained upon total

release. Measurement start time was chosen at 0 s and the number of flashes were 10 flashes per well and cycle.

Kinetic window	1	2	3
Number of cycles	3	3	5
Cycle time (s)	120	600	1200

Table 2.2: Kinetic window protocol for pDNA release experiments

2.2.11. Vesicle-Vesicle Interaction: Membrane Lipid Mixing Assays

2.2.11.1. Octadecyl Rhodamine B (R18) Dequenching

To investigate the fusion between the intact anionic liposomes used previously to trigger pDNA release from lipoplexes, a method has been developed based on the relief of fluorescence self quenching. In this assay, a fluorescent lipid like probe octadecyl rhodamine B chloride (R18), is inserted into the lipoplexes. The fluorescently labelled lipoplexes were prepared by conventional mixing or detergent dialysis techniques by addition of (OD R18) ethanolic solution of to the cationic lipid organic solution. At a sufficiently high concentration of 5 mol% with respect to the total lipid content, an efficient self-quenching of the fluorescence occurs. Fusion of labelled lipoplexes with anionic liposomes membranes will result in dilution of the probe and concomitant relief of the self-quenching. The increase in the fluorescence observed is taken as a measure of fusion. Once inserted in the lipidic membrane, the probe does not dissociate from the membranes by either spontaneous transfer of the free monomer through the aqueous phase or by collional transfer.

Lipoplexes were prepared with 1.5 µmole of cationic lipid DOTAP or Dc-cholesterol, mixed with 100 µg plasmid DNA at (ρ) of 5 and diluted in 20 mM HEPES, 145 mM NaCl buffer at pH 7.4. Labelled cationic lipoplexes were pipetted into a 96 well plate, and treated with unlabeled negatively charged lipids at 2 and 4 fold molar excess of negative charges at 25 °C. Fluorescence increase occurred upon relief of self-quenching, was monitored for approx. 7000 s using a microplate reader (Fluostar optima, BMG Labtechnologies, D-Offenburg, Germany). Fluorescence intensity was monitored as a function of time with an excitation wavelength λ_{ex} 544 nm and an emission wavelength λ_{em} 590 nm at 25 °C. Measurements were taken according to kinetic window as stated in Table 2.2, yielding fluorescence values at the onset (F_0) and during the reaction (F_t). Parallel measurements of samples containing 200 mM OGP were averaged and used as a value for the fluorescence after infinite dilution (F_{OGP}). The relative total fluorescence change was calculated using the following equation (Jun and Wickner, 2007):

$$\Delta F_t / F_{OGP} = (F_t - F_0)/F_{OGP}$$

Where F_t is the fluorescence intensity at a certain time point, F_0 the residual fluorescence before addition of anionic liposomes, and F_{OGP} the maximum fluorescence obtained after complete lipid mixing.

Fusion dependent change of fluorescence was calculated by subtracting fusion independent fluorescence change values represented by a sample containing only labelled donor lipoplexes from total fluorescence change values.

2.2.11.2. Fluorescence Resonance Energy Transfer (FRET)

FRET was used to assess lipid mixing after the addition of anionic liposomes to lipoplexes according to a well established fluorescence resonance energy transfer assay (Struck et al., 1981) using the probe dilution method. Briefly, cationic lipoplexes were prepared by mixing and detergent dialysis methods. They were labelled with 0.5% NBD-PE and 0.5% rhodamine-PE (Rh-PE). Negatively charged liposomes were prepared at 10 mM lipid concentration without fluorescent probes. Labelled cationic lipoplexes were placed in a 96 well plate, and were treated with unlabeled negatively charged lipids at 25 °C at a 2 fold excess of negative charges. Fluorescence intensity was monitored as a function of time with λ_{ex} 460 nm, λ_{em} 530 nm at 25 °C. Fluorescence was also measured for samples with negatively charged liposomes which were mixed the presence of 200 mM OGP. This intensity was then used for normalization of measurements. In the probe dilution method, the residual fluorescence of the labelled vesicles containing 0.5 mol % each of NBD-PE and Rh-PE was taken as 0% of maximum fluorescence. Samples used for the calibration of the fluorescence to 100% maximum fluorescence (F_{max}) were taken for those treated with 200 mM OGP. Complete intermixing of all bilayers upon fusion would be expected to result in a membrane containing 0.3 mol% each of the two fluorescent phospholipids, which was taken as the theoretical maximum fluorescence. NBD fluorescence measurements were continuously monitored, using an excitation wavelength of λ_{ex} 460 nm and an emission wavelength λ_{em} 530 nm.

The transfer efficiency (*E*) can be determined by steady state measurements of the extent of donor (D) NBD fluorescence de-quenching due to the acceptor (Struck et al., 1981).

$$E = 1 - [F_{DA} / F_D]$$

Where, F is the fluorescence intensity in the absence ($\mathbf{F}_D$) and presence ($\mathbf{F}_{DA}$) of the acceptor.

2.2.12. Cryo-Transmission Electron Microscopy (Cryo-TEM)

The morphology of lipoplexes as well as the structures during pDNA release was determined by transmission cryo-TEM. For cyro-TEM experiments, DOTAP lipoplexes were mixed with different anionic liposomes at 4 fold molar excess of negatively charged lipids. One minute and 1 h after

mixing, samples were prepared for visualization as previously adapted (Bunjes et al., 2007): A 3 µl droplet of the lipoplex/anionic liposome suspension is deposited on an electron microscopy copper grid with perforated carbon film (Quantifoil R 1.2/1.3) (Quantifoil micro tools, Jena, Germany). After removing the excess solution with a filter paper, the thin layer of water is plunged rapidly into liquid ethane at -178 °C in a cryobox (Zeiss, Oberkochen). Excess ethane was removed with a piece of filter paper. The grid, kept under liquid nitrogen, is then transferred into a cryo-holder (Gatan 626, Pleasanton, CA and München, Germany) maintained at -170 °C, which, in turn, is introduced into the TEM for observation at -170 °C. To limit beam damage, the specimens are observed with a limited amount of electrons using the low-dose technique. Images were obtained under cryogenic conditions and investigated at 120 kV in a Philips CM 120 electron microscope. The micrographs were generated by a Tietz-Fast Scan CCD camera.

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CHAPTER 3

Physicochemical Characterization of Lipoplexes Prepared by Detergent Dialysis Technique

3. Physicochemical Characterization of The Lipoplexes

Abstract

The preparation of lipoplexes consisting of cationic lipid/plasmid DNA (pDNA) which form as a result of a self assembly process in the presence of the non-ionic detergent N-octyl-beta-D-glucopyranosid (OGP) has been investigated (Reimer et al., 1995; Bally et al., 1997; Zhang et al., 1997). This assembly is dependent on the formation of a hydrophobic cationic lipid/pDNA complex intermediate. It was assumed that the interaction between cationic lipid/pDNA assemblies is mediated by hydrophobic interactions rather than charge-charge interactions (Zhang et al., 1997).

The detergent dialysis technique used in the present work is based on the preparation of mixed micelles containing the non-ionic detergent N-octyl-beta-D-glucopyranosid OGP and cationic lipids. When these micelles were mixed with plasmid, defined particles formed spontaneously. Consecutive detergent removal leads to the formation of lipid/plasmid DNA lipoplexes. This technique was shown to produce highly stable lipoplexes against nucleases compared to those produced by mixing technique (Guo et al., 2000; Xu et al., 2001).

In this chapter, lipoplexes were prepared using DOTAP and DC-cholesterol cationic lipids at N/P ratios 4 and 5 in the presence of 200 mM OGP based on earlier investigations.

The physicochemical properties of the dialysed lipoplexes were investigated and correlated to that of mixed lipoplexes. The mixed and dialysed lipoplexes were characterized by laser light scattering to determine the influence of OGP on the hydrodynamic diameter and the surface charge of the lipoplexes. Additionally, to characterize these lipid/based pDNA formulations, we have used an approach similar to that described by Xu and Szoka (1996), in which anionic liposomes containing composition that mimic the cytoplasmic facing monolayer of the plasma membrane (e.g. phosphatidylserine) rapidly release pDNA from the complex. The lipoplexes destabilize by a flip flop process of the anionic lipids from the cytoplasmic facing monolayer of the endosomal membrane into the inner monolayer, which laterally diffuse into the complex and form a charge neutral ion-pair with the cationic lipids. In other report, minor cellular lipids like oleic acid (OA) possessed strong pDNA releasing activity (Tarahovsky et al., 2004). In this study, the PicoGreen® assay, as pDNA marker, was used to assess pDNA condensation state.

Lipoplexes prepared by detergent dialysis technique showed a pronounced increase in hydrodynamic diameters compared to mixed lipoplexes. The increase in particle size was better observable in DOTAP/pDNA than DC-cholesterol/pDNA lipoplexes. The increase in hydrodynamic diameter was accompanied by remarkable decrease in the zeta potentials. This was obvious in both lipoplexes at N/P ratios examined.

Corresponding to the physicochemical characteristics, this was supplemented by pDNA condensation as measured using PicoGreen® assay. The strong pDNA compaction of dialysed lipoplexes showed lower pDNA de-condensation capability compared to the mixed lipoplexes when anionic lipids were applied.

In conclusion, the preparation technique influenced the physicochemical characteristics of produced lipoplexes in terms of hydrodynamic diameter, zeta potential as well as pDNA compaction. The effect of OGP on pDNA compaction was well characterized by a significantly lower pDNA release from dialysed lipoplexes compared to mixed lipoplexes regardless to the cationic lipid used to condense pDNA and the N/P ratios used.

3.1. *Size of Lipoplexes*

The hydrodynamic diameters of mixed and dialysed lipoplexes of DOTAP and DC-cholesterol were measured in order to compare the effect of OGP on various mixing ratios between pDNA and cationic lipids.

The hydrodynamic diameter of the lipoplexes was determined by photon correlation spectroscopy. For these measurements, mixed DOTAP/pDNA and DC-cholesterol/pDNA lipoplexes were prepared with plasmid DNA at different N/P ratios at 4 and 5 in 20 mM HEPES/145 mM NaCl buffer solution at pH 7.4. Mixing of pDNA and cationic lipids leads to complex formation based on electrostatic interactions. Dialyzed lipoplexes were formed by an alternative technique complexing pDNA and cationic lipids in the presence of 200 mM non-ionic detergent OGP followed by dialysis. For details of preparation see chapter 2 Materials and Methods section *2.2.2*.

The optimal ratio found for *in-vitro* transfection is in the range of 3.6 to 9 (cationic lipid: pDNA, nmol: μg) depending on the types of cationic liposomes, and cell types used (Liu and Song, 1998).

Under the chosen conditions pDNA and cationic lipids were prepared with the following cationic lipid/pDNA molar ratio, (ρ) = L_C/D = (moles of cationic lipid)/ (moles of pDNA bases) of 4 and 5. According to previous studies, this could be a condition in which the majority of pDNA molecules should be complexed (Gershon et al., 1993; Lee et al., 2004).

Hydrodynamic diameter was found to be dependent on preparation technique of lipoplexes, cationic lipid used to condense pDNA and the N/P ratio.

Dialysed DOTAP/pDNA lipoplexes had larger diameters than mixed lipoplexes. More specifically, the DOTAP/pDNA lipoplexes formed by the mixing technique without OGP showed comparable hydrodynamic diameters of 218.2 ± 8 nm and 297.5 ± 20.2 nm at N/P 4 and 5, respectively (Figure 3.1 A). Formation of DOTAP/pDNA lipoplexes in the presence of 200 mM OGP resulted in an increase in size to 1176 ± 471.09 nm and 601.3 ± 311.1 nm at N/P 4 and 5, respectively. Additionally, the polydispersity indices (PDI) of the size measurements as a marker for particle size distribution width were diverse. PDI showed a wide size distribution width for both mixed and dialysed lipoplexes demonstrating a heterogeneous size distribution. Increasing the N/P ratio from 4 to 5 improved the polydispersity (PDI) of produced DOTAP/pDNA lipoplexes prepared by mixing technique.

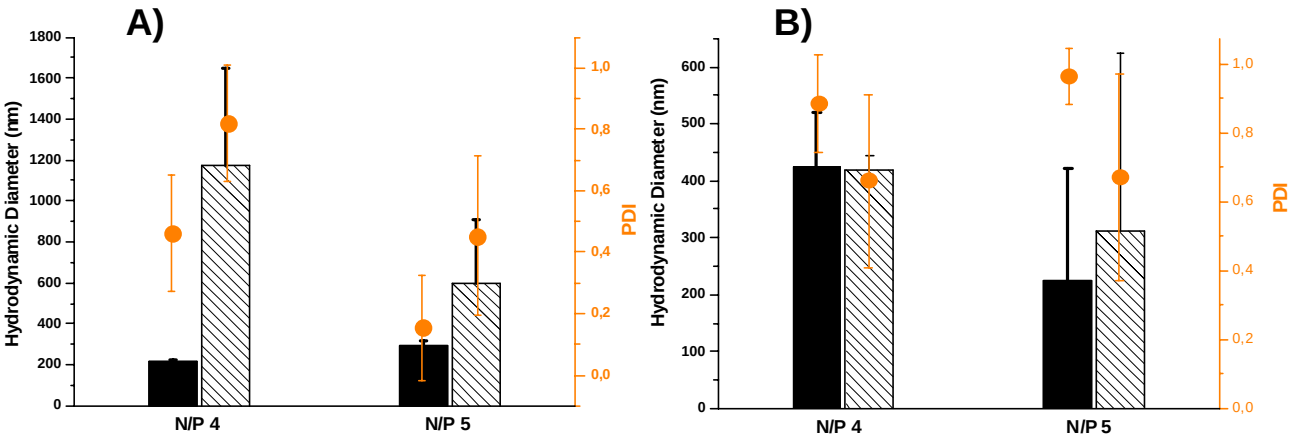

Figure 3.1: Influence of OGP on the hydrodynamic diameter of lipoplexes at different N/P ratios. The effect of OGP concentrations (200 mM) on the hydrodynamic diameter at N/P ratio 4 and 5 of mixed (striated bars) and dialysed (solid bars) DOTAP (A) and DC-cholesterol (B) was measured by photon correlation spectroscopy in three independent experiments. Results are given as mean +/- standard deviation.

In contrast, the effect of OGP (200 mM) on hydrodynamic diameter of DC-cholesterol/pDNA at N/P 4 and 5 was not observed compared to the same mixed lipoplexes (Fig. 3.1 B). DC-cholesterol/pDNA lipoplexes showed comparable hydrodynamic diameters of 424.5 ± 96.5 nm (mixed) and 418.9 ± 25.8 nm (dialysed) at N/P 4, while a slight increase in the hydrodynamic diameters observed for dialysed (311 ± 312.8 nm) compared to mixed (224.4 ± 196.2 nm) DC-cholesterol/pDNA lipoplexes at N/P 5 (Fig. 3.1 B).

The PDI of mixed and dialysed lipoplexes exhibited high values of 0.89 ± 0.14 (mixed) and 0.96 ± 0.08 (dialysed) at N/P 4 the condition which indicates heterogeneity of the lipoplexes. The increase in PDI of DC-cholesterol/pDNA lipoplexes was accompanied by a bimodal peak profile suggesting aggregation of the lipoplexes, both in case of mixed and dialysed lipoplexes.

Based on the structures of cationic headgroups, DC-cholesterol contains a tertiary amine while DOTAP cationic lipid contains quaternary amine. The tertiary amine of DC-cholesterol is non-ionized at neutral to alkaline pH shows a pKa of 8 (Zuidam and Barenholz, 1997) compared to the quaternary amine of DOTAP that is ionized at all pH ranges (Ajmani and Hughes, 1999). It was assumed that interaction of cationic DC-cholesterol vesicles with negatively charged plasmid DNA will lead to further protonation of the DC-cholesterol tertiary amine, due to lowering of the pH of the surface membrane by the charged phosphates (Zuidam and Barenholz, 1998). Consequently, this leads to more free cationic interaction with the pDNA wrapped DC-cholesterol vesicles by attraction forces. This interaction may produce large aggregates with a high zeta potential value as shown below for mixed DC-cholesterol/pDNA lipoplexes.

The heterogeneous size distribution was previously reported for lipoplexes prepared by mixing technique (Ciani et al., 2004). This might be due to the aggregation of the intact cationic liposomes wrapped by pDNA by electrostatic attraction forces. It has been also demonstrated, for example, that pDNA binding to liposomes can provoke significant disruption in liposome structure (Sternberg et al., 1994; Rädler et al., 1997; Wasan et al., 1999).

Conclusively, in the case of DOTAP/pDNA lipoplexes, detergent dialysis technique showed an increase in size of the lipoplexes presumably by aggregation enhancement. The effect was pronounced at N/P 4 than at N/P 5. In case of DC-cholesterol/pDNA lipoplexes, OGP had a lower influence on the resulting lipoplex size.

3.2. *Zeta Potential of Lipoplexes*

Formation of lipoplex is mediated through simple electrostatic interactions, typically between positively charged cationic liposomes and the phosphate groups of pDNA (Zuidam and Barenholz, 1998; Ciani et al., 2004). The ratio of cationic lipid to pDNA has been shown to be one of the most important factors affecting the transfection efficiency.

These systems are formulated in a manner whereby the resulting cationic lipid/pDNA lipoplexes exhibit a positive charge that facilitates association with the negatively charged cell surface domains (Stamatatos et al., 1988; Wong et al., 1999; Liang and Chou, 2009). Zeta potential is an indirect measurement of the lipoplex surface charge, and it can be used to evaluate the extent of interaction of the cationic lipid surface charges with the anionic charges of pDNA (Eastman et al., 1997; Perrie and Gregoriadis, 2000; Perrie et al., 2001; Ma et al., 2007).

Figure 3.2 shows the zeta potential values of mixed and dialysed lipoplexes, as measured by LDA, as a function of: (i) the method of preparation, (ii) the type of cationic lipid used to condense pDNA and (iii) the mixing N/P ratios. Mixed DOTAP/pDNA lipoplexes showed zeta potentials of 48.6 ± 4 mV (N/P 4) and 44.64 ± 6.3 mV (N/P 5). The presence of the OGP detergent during lipoplexes assembly caused a reduction in zeta potential upon detergent withdrawal (Fig. 3.2 A). Zeta potentials of 30.2 ± 3.1 mV (N/P 4) and 36.65 ± 4.8 mV (5 N/P) were recorded for dialysed DOPAP/pDNA lipoplexes. The reduction in zeta potential at N/P 4 than at N/P 5 the condition indicates higher cationic DOTAP lipids are involved in pDNA neutralization.

A similar pattern in the zeta potential values as a function of preparation technique and mixing ratios was also observed for DC-cholesterol/pDNA lipoplexes prepared by detergent dialysis technique at the same N/P ratios (Fig. 3.2 B). In general, the mixing technique resulted in the formation of positively charged lipoplexes. Nevertheless, the zeta potentials of DC-cholesterol/pDNA lipoplexes were lower than the values of DOTAP/pDNA lipoplexes prepared by mixing technique. The increase in the N/P ratio from 4 to 5 barely changed the zeta potential of mixed lipoplexes. Mixed DC-cholesterol/pDNA lipoplexes exhibited zeta potential values of 36.02 ± 2.1 mV and 36.76 ± 3.5 mV at N/P ratio 4 and 5, respectively. In contrast, the presence of OGP during lipoplex preparation

produced lipoplexes with reduced zeta potentials. These values were 26.1 ± 3.6 mV and 25.32 ± 0.95 mV for N/P ratio 4 and 5, respectively.

In accordance to previous studies, DOTAP/pDNA and DC-cholesterol/pDNA lipoplexes prepared by conventional mixing technique showed zeta potential of + 48 mV and +39 mV, respectively (Ciani et al., 2004). This was in the line to our zeta potential values for mixed lipoplexes.

The increase in DOTAP/pDNA lipoplexes size due to OGP effect was correlated with lower zeta potentials.

Conclusively, the detergent dialysis may influence the interactions between pDNA and transfection compounds. The presence of non-ionic detergent during cationic lipid/pDNA assembly generally affects the particle size and the zeta potentials of the produced lipoplexes.

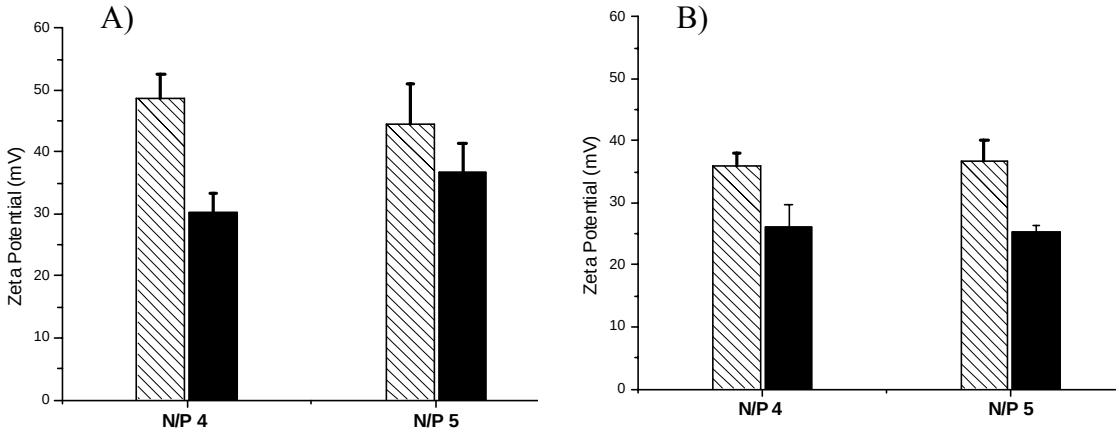

Figure 3.2: Effect of OGP (200 mM) on the zeta potential of different lipoplexes at different N/P ratios. The zeta potential of mixed (striated bars) and dialysed (solid bars) DOTAP/pDNA (A) and DC-cholesterol/pDNA (B) lipoplexes at two different N/P ratios 4 and 5 was measured by laser Doppler anemometry in three independent experiments. Results are shown as mean +/- standard deviation.

3. 3. pDNA Release

Szoka and co-workers reported that different types of anionic lipids were equally effective in pDNA release. In the meantime, twofold excess of anionic lipid was sufficient to release 80-90 % of the lipoplex pDNA (Xu and Szoka, 1996; Zelphati and Szoka, 1996). A similar process of lipid exchange between lipoplexes and cytoplasmic membranes resulting in pDNA release into the cytoplasm was later observed in cultured cells (Wrobel and Collins, 1995; Noguchi et al., 1998; Nakanishi and Noguchi, 2001). Furthermore, intermixing of endosomal membrane lipids with lipoplex lipids seems to be an important step in the transfection process.

We applied the PicoGreen® assay as pDNA fluorescent probe to examine the pDNA condensation state in the mixed and dialysed lipoplexes as a function of time. This was taken as a measure of pDNA release. The pDNA release was induced by addition of different negatively charged liposomes.

The results are presented as plots of the percentage of pDNA released from cationic DOTAP/pDNA and DC-cholesterol/pDNA lipoplexes after incubation with negatively charged DOPS or oleic acid containing liposomes. Several negatively charged liposomes were used for the experiments: (i) DOPS/DOPC (3:7), (ii) DOPS: DOPE: DOPC (2.5:2.5:5), (iii) oleic acid (OA)/DOPE (3:7), (iv) OA/DOPC (3:7) and (v) OA/DOPE/DOPC (2.5:2.5:5).

The cationic lipid/pDNA ratio influenced both lipoplex formation and pDNA release after addition of negatively charged lipid (Pantazatos and MacDonald, 2003; Tarahovsky et al., 2004; Koynova and MacDonald, 2007). Therefore, the lipoplexes were prepared at N/P ratios 4 and 5. Lipoplexes were equilibrated for 30 min before addition of the negatively charged liposomes in case of mixed lipoplexes and equilibrated in 200 mM OGP followed by detergent dialysis in case of dialysed lipoplexes.

The negatively charged liposomes were added to the cationic lipoplexes at $R = 4$ where $R = A/Lc$ (moles of anionic lipid/moles of cationic lipid). pDNA release was estimated by an increase of PicoGreen® fluorescence after pDNA displacement from lipoplexes by negatively charged liposomes. pDNA release was expressed as percentage of pDNA released as previously described in chapter 2 Materials and Methods section *2.2.10*.

3.3.1. pDNA Release from DOTAP/pDNA and DC-cholesterol/pDNA Lipoplexes by DOPS Containing Liposomes

3.3.1.1. DOTAP/pDNA Lipoplexes

For DOTAP/pDNA lipoplexes, the mixing technique always demonstrated a higher pDNA release by anionic DOPS/DOPE/DOPC and DOPS/DOPC liposomes compared to dialysed lipoplexes. Even after more than 2 h, only a part of the pDNA was released from dialysed lipoplexes. Experiments using liposomes with and without DOPE resulted in different release profiles for the DOTAP/pDNA lipoplexes tested. Furthermore a higher N/P ratio of 5 resulted in a higher pDNA release compared to

N/P 4 in case of DOPS/DOPC liposomes while DOPS/DOPE/DOPC liposomes were efficient to displace pDNA from DOTAP/pDNA lipoplexes at N/P 4 than N/P 5.

Following addition of DOPS/DOPC containing liposomes to mixed DOTAP/pDNA lipoplexes at N/P 5 (Fig. 3.3 A), pDNA release was not considerably different as compared to the pDNA release by DOPS/DOPE/DOPC liposomes (Fig.3.3 B).

DOPS/DOPE/DOPC liposomes induced a maximum of 40.5 ± 0.7 % pDNA release compared to 38.65 ± 1.1 % for DOPS/DOPC liposomes. A slight difference in the initial release rate could be detected which is more steep in case of DOPE containing liposomes than in case of only DOPS/DOPC liposomes indicating higher initial pDNA release rate in the first phase (Fig. 3.3).

In contrast, the maximum pDNA release from dialysed DOTAP/pDNA lipoplexes exhibited only 6.1 ± 0.2 and 4.0 ± 0.4 % for DOPS/DOPC and DOPS/DOPE/DOPC, respectively (Fig. 3.3). A small amount of pDNA from dialysed lipoplexes could be detected immediately after the addition of negatively charged liposomes. We assume that this small amount of uncondensed pDNA was attached to lipoplex surface and hence was available to PicoGreen® dye intercalation, but the mean pDNA is still retarded by cationic lipid aggregate.

A considerable difference in pDNA displacement was shown at N/P ratio 4 than 5. This was observed for both mixed and dialysed DOTAP/pDNA lipoplexes treated with DOPS/DOPE/DOPC liposomes (Fig. 3.3).

DOPS/DOPC liposomes exhibited greater pDNA release efficiency at N/P 5 ratio than observed at N/P 4. At N/P 4, mixed DOTAP/pDNA lipoplexes showed 26.9 ± 1.1 % pDNA released while at N/P 5 pDNA release was 38.65 ± 1.1 % (Fig. 3.3 A).

A difference in the pDNA release efficiency from DOTAP/pDNA lipoplexes was observed when DOPS/DOPE/DOPC liposomes were used compared to DOPS/DOPC liposomes. As seen in Figure 3.3 B, mixed DOTAP/pDNA lipoplexes showed complete pDNA release at N/P 4, while exhibited only 40.5 ± 0.7 % at N/P 5.

The effect of DOPE to induce more pDNA release at N/P 4 than N/P 5 could be referred to its ability to form fusion with DOTAP lipid in the lipoplex.

A similar effect of DOPS/DOPE/DOPC liposomes to displace pDNA release was also observed in case of dialysed DOTAP/pDNA lipoplexes at N/P 4 than N/P 5. Dialysed DOTAP/pDNA lipoplexes showed 21.2 ± 2.65 % (N/P 4) and 4.0± 0.4 % (N/P 5) pDNA released (Fig. 3.3 B).

3.3.1.2.. DC-cholesterol/pDNA Lipoplexes

Dialysed DC-cholesterol/pDNA lipoplexes demonstrated in all cases a lower pDNA release compared to mixed lipoplexes. This corresponds to the results for the DOTAP/pDNA lipoplexes described in the last section. In contrast, the effect of DOPE on pDNA release from DC-cholesterol/pDNA lipoplexes was lower than observed for DOTAP/pDNA lipoplexes. Furthermore, higher N/P ratios showed higher pDNA release.

Mixed DC-cholesterol/pDNA lipoplexes showed 23.5 ± 0.18 and 29 ± 1.1% pDNA released at N/P of 4 and 5, respectively when DOPS/DOPC liposomes were used (Fig. 3.4 A). A similar final extent of pDNA released was observed when these lipoplexes were treated with DOPS/DOPE/DOPC liposomes at N/P 4 (24.5 ± 1.3%) and (32.6 ± 0.75 %) at N/P 5 (Fig. 3.4 B).

Like dialysed DOTAP/pDNA lipoplexes, dialysed DC-cholesterol/pDNA lipoplexes exhibited also a low pDNA release pattern. DOPS/DOPC liposomes exerted a slight increase in the extent of pDNA released at N/P 5 (7.5 ± 0.1%) than at N/P 4 (3 ± 0.03 %). While, dialysed DC-cholesterol showed only 3.1 ± 0.1 % (N/P 4) and 3.7 ± 0.2 % (N/P 5) pDNA released when DOPS/DOPE/DOPC liposomes were applied (Fig. 3.4).

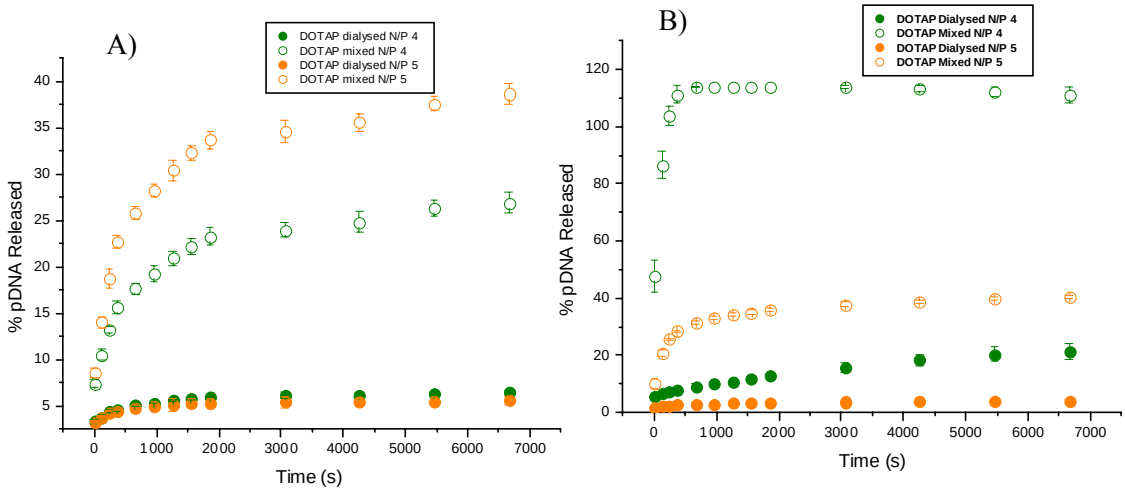

Figure 3.3: Time-dependent effect of N/P ratio and preparation technique on the pDNA release from DOTAP/pDNA lipoplexes using DOPS/DOPC (3:7) (A) and DOPS/DOPE/DOPC (1:1:2) (B) liposomes. DOTAP/pDNA lipoplexes prepared at N/P 4 (dialysed (●); mixed (○)) and N/P 5 (mixed (●) and mixed (○)) were treated with DOPS at R=4. PicoGreen® solution was added to 2µg/ml pDNA/ DOTAP lipoplexes, and the increase of PicoGreen® fluorescence was monitored over time at pH 7.4 as described in the Materials and Methods section *2.2.10.* Data were shown as mean +/- standard deviation of three independent experiments.

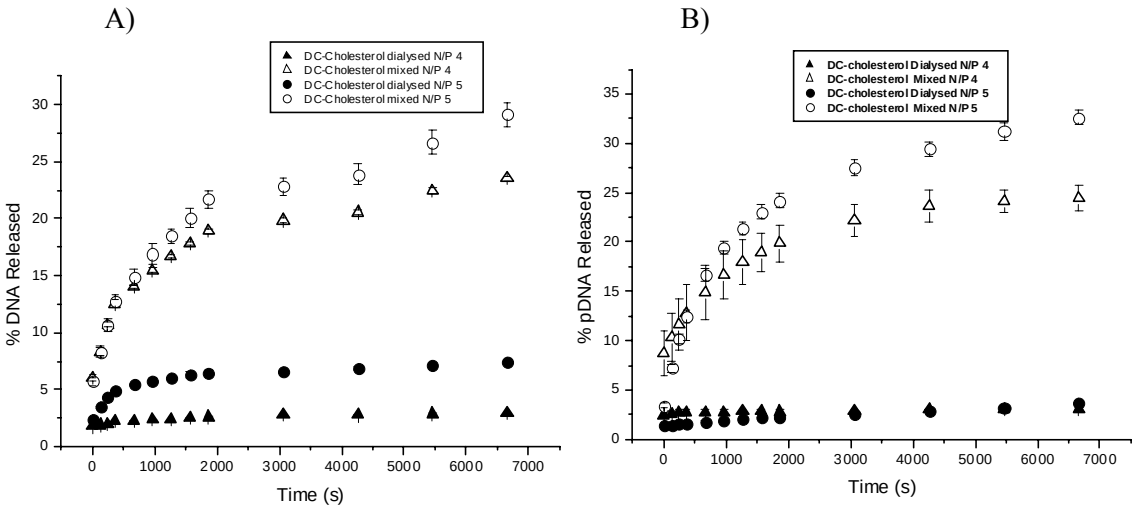

Figure 3.4: Time-dependent effect of N/P ratio and preparation technique on the pDNA release from DC-cholesterol/pDNA lipoplexes using DOPS/DOPC (A) and DOPS/DOPE/DOPC (B) liposomes. DC-cholesterol/pDNA lipoplexes were treated with DOPS/DOPC (3:7) and with DOPS/DOPE/DOPC (1:1:2) at N/P 4 (dialysed (▲); mixed (Δ)) and N/P 5 (dialysed (●) and mixed (○)) at R=4. PicoGreen® solution was added to 2µg/ml pDNA/ of DC-cholesterol lipoplexes, and the increase of PicoGreen® fluorescence was monitored over time at pH 7.4 as described in the Materials and Methods section *2.2.10*. Data were shown as mean +/- standard deviation of three independent experiments.

3.3.2. pDNA Release from DOTAP/pDNA and DC-cholesterol/pDNA Lipoplexes by oleic acid (OA) Containing Liposomes

Oleic acid is considered as a minor cellular lipid component of the biological membranes (Epand et al., 1991; Tarahovsky et al., 2004). In previous work, oleic acid was a strong pDNA releaser from *o*-ethyl-dioleoylphosphatidylcholinium (EDOPC) lipoplexes (Tarahovsky et al., 2004). In this study, negatively charged oleic acid was used in combination with different phospholipids to exert pDNA release from DOTAP/pDNA and DC-cholesterol/pDNA lipoplexes (Fig. 3.5 and Fig. 3.6).

3.3.2.1. DOTAP/pDNA Lipoplexes

Changing the type of the anionic lipid did affect the extent of pDNA release from DOTAP/pDNA lipoplexes. Mixed DOTAP/pDNA lipoplexes were characterized by a higher pDNA release in the presence of oleic acid (OA) compared to dialysed lipoplexes. Lipoplexes prepared at N/P ratio 5 always were found to release more pDNA than lipoplexes at N/P 4. Additionally, the oleic acid

capability to release pDNA from DOTAP/pDNA lipoplexes increased in the range from DOPE < DOPC < DOPE/DOPC.

As shown in Figure 3.5 A and Fig. 3.5 B, the efficiency of oleic acid to exert pDNA release from DOTAP/pDNA lipoplexes was varied depending on the phospholipid compositions. When OA/DOPC liposomes were used, oleic acid exhibited an ability to release pDNA to about 36 ± 0.4 % (mixed lipoplexes) and a lower pDNA release (22.1 ± 0.7 %) could be detected at N/P 5 when OA/DOPE liposomes was used.

OA/DOPE/DOPC liposomes showed stronger pDNA release in terms of the percent (45.8 ± 1.3 %) and the release rate, Figure 3.5 C, compared to the pDNA released by DOPS/DOPE/DOPC liposomes (40.5 ± 0.7 %).

The initial rate and extent of the pDNA release were depended on composition of the phospholipids used to initiate pDNA release. The highest pDNA release could be found for OA/DOPE/DOPC liposomes (45.8 ± 1.3 %) for DOTAP/pDNA lipoplexes at N/P 5 (Fig. 3.5 C).

For dialysed DOTAP/pDNA lipoplexes, the rate of pDNA release was low for all oleic acid lipids combinations tested.

Figure 3.5 shows the effect of OA/DOPC liposomes (4.8 ± 0.1%); OA/DOPE (3 ± 0.3 %) and OA/DOPC/DOPE (4.8 ± 0.4 %) to release pDNA from dialysed DOTAP/pDNA lipoplexes at N/P 5 was observed.

The OA capability to release pDNA in presence of DOPE was lower than OA/DOPC liposomes. In contrast, OA ability to release pDNA from DOTAP/pDNA lipoplexes in presence of DOPE and DOPC improved the OA pDNA release capability.

The neutral lipid DOPE adopts non-bilayer inverted hexagonal (H_{II}) phase in isolation. To stabilize the Lα phase of DOPE, anionic lipids such as oleic acid was previously used in its ionized state (Düzgüneş, 2004). The lower capability of oleic acid/DOPE to release pDNA from DOTAP/pDNA lipoplexes may be due its stabilizing effect to DOPE into Lα phase bilayer. As a consequence, its partition from the OA/DOPE vesicles to form DOTAP/OA ion pair is not enhanced.

The N/P ratio influenced the maximum extent of pDNA release after addition of negatively charged liposomes. In all cases, the rate of pDNA release estimated from DOTAP/pDNA lipoplexes at N/P 4 was lower than that at N/P 5(Fig. 3.5 A, B and C).

The free cationic lipids around the formed lipoplexes are considered to be necessary to initiate pDNA release through the ion-pair formation with anionic lipids. At lower N/P ratio, the excess cationic lipid is low; therefore, the lipids bound to pDNA are not able to form neutralized ion-pair with anionic lipid which is a prerequisite to initiate pDNA release from lipoplexes (Pantazatos and MacDonald, 2003; Koynova and MacDonald, 2007).

In the line with our data, pDNA release from DOTAP/ODNs previously showed that the pDNA release was highest at excess positive charge of the complexes (Jääskeläinen et al., 1998).

From a previous report, the pDNA release from EDOPC lipoplexes was investigated (Koynova and MacDonald, 2007). They showed that lipoplexes with excess cationic lipids rapidly released their pDNA content upon addition of anionic liposomes. While, in the case of the lipoplexes with excess pDNA, the release of pDNA was quite inefficient even after 1 h of incubation with the same anionic lipids.

3.3.2.2. DC-cholesterol/pDNA Lipoplexes

As observed in the experiments described above, mixed DC-cholesterol based lipoplexes demonstrated higher pDNA release than dialysed lipoplexes in the presence of OA containing liposomes. Again, N/P ratio of 5 resulted in a higher pDNA release than at N/P ratio 4. Independent from the composition of the anionic liposomes (OA/DOPC, OA/DOPE and OA/DOPE/DOPC), the pDNA release from mixed DC-cholesterol lipoplexes was comparable for each liposomes composition (Fig. 3.6).

Mixed DC-cholesterol/pDNA lipoplexes treated with oleic acid containing liposomes exhibited a considerable pDNA released compared to DOTAP lipoplexes.

A percentage of 43 ± 0.7 % pDNA was released from mixed DC-cholesterol/pDNA lipoplexes at N/P 5 when a mixture of OA/DOPC was used (Fig. 3.6 A). In contrast, mixed DOTAP/pDNA lipoplexes showed only 36 ± 0.4 % pDNA released for the same negatively charged lipid composition (Fig. 3.5 A).

Unlike the effect on DOTAP/pDNA lipoplexes, OA/DOPE liposomes showed pDNA release from mixed DC-cholesterol/pDNA lipoplexes as twice as that observed in case DOTAP/pDNA lipoplexes at N/P 5 (Fig. 3.5 B and Fig. 3.6 B).

OA/DOPE/DOPC liposomes showed the highest capability to exert pDNA release in both lipoplexes (Fig. 3.5 C and Fig. 3.6 C). The effect of OA/DOPE to exert pDNA release from DC-cholesterol/pDNA lipoplexes was as equal as the effect of other lipid combinations (Fig. 3.6 A-C)

Like the other dialysed lipoplexes, the rate of pDNA release from dialysed DC-cholesterol/pDNA lipoplexes was considerably low. pDNA release in terms of method of preparation and N/P ratios, OA exhibited pDNA percentages of 6.5 ± 0.48, 5.3 ± 0.19 and 7.7 ± 1.16 % pDNA released for DOPC; DOPC/DOPE and DOPE containing oleic acid at N/P 5 respectively(Fig. 3.6).

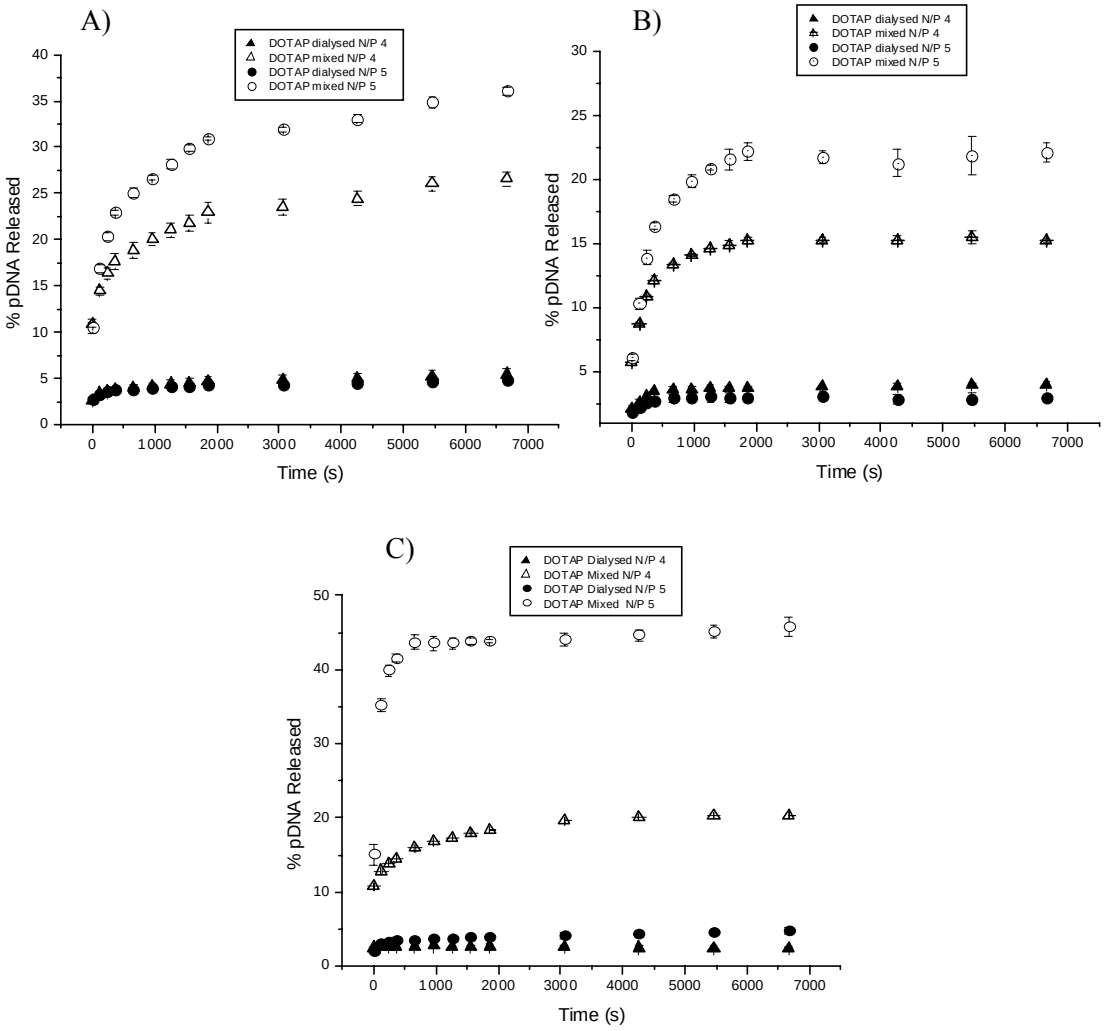

Figure 3.5: Time-dependent effect of N/P ratio and preparation technique on the pDNA release DOTAP/pDNA lipoplexes using various oleic acid (OA) liposomes. DOTAP/pDNA lipoplexes were treated OA/DOPC (3:7) (A); OA/DOPE (3:7) (B) and OA/DOPE/DOPC (1:1:2) (C) at N/P 4 (dialysed (▲); mixed (Δ)) and N/P 5 (dialysed (●) and mixed (○)) at R=4. PicoGreen® solution was added to 2µg/ml pDNA/ of DOTAP lipoplexes, and the increase of PicoGreen® fluorescence was monitored over time at pH 7.4 as described in the Materials and Methods section *2.2.10*. Data were shown as mean +/- standard deviation of three independent experiments.

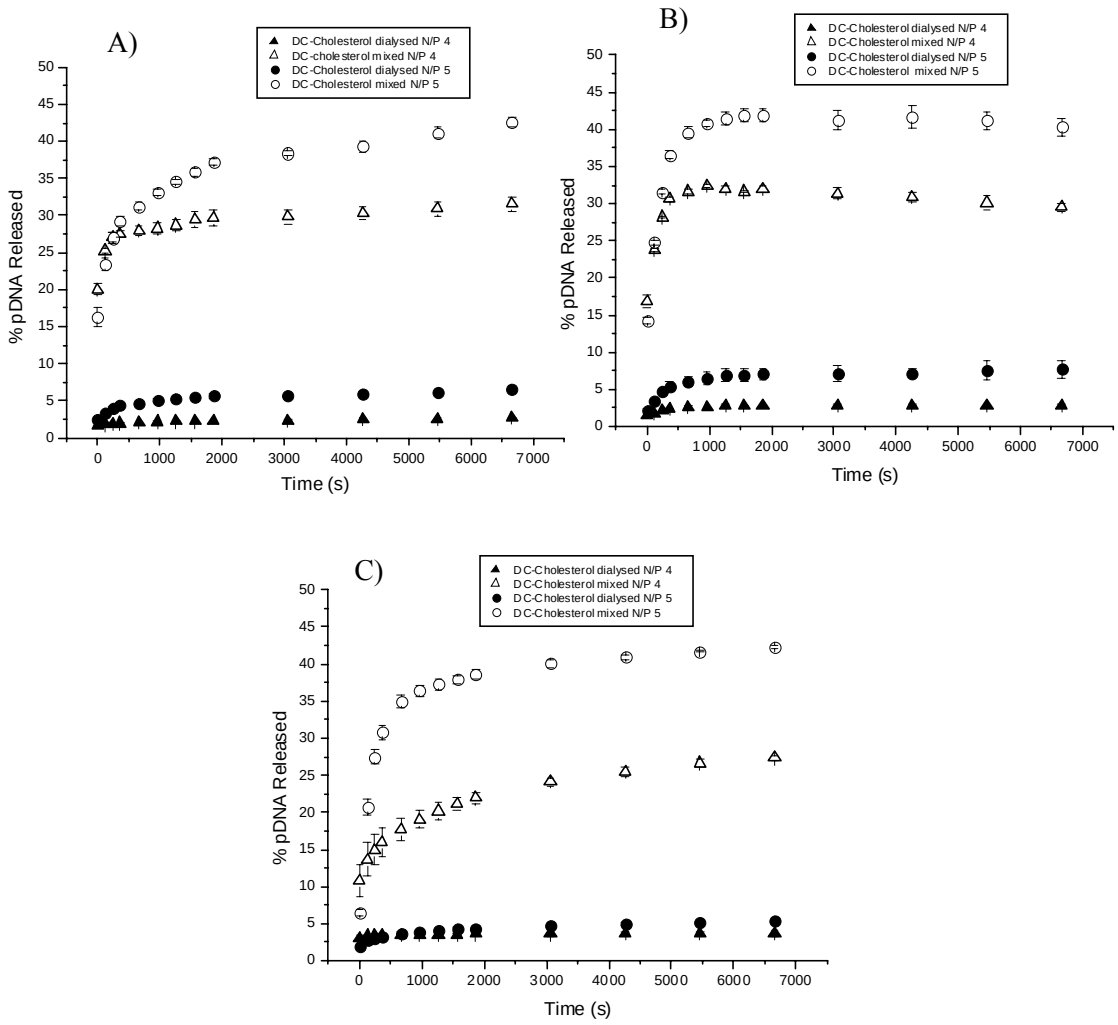

Figure 3.6: Time-dependent effect of N/P ratio and preparation technique on the pDNA release DC-cholesterol/pDNA lipoplexes using various oleic acid (OA) liposomes. DC-cholesterol/pDNA lipoplexes were treated OA/DOPC (3:7) (A), OA/DOPE (3:7) (B) and OA/DOPE/DOPC (1:1:2) (C) at N/P 4 (dialysed (▲); mixed (Δ)) and N/P 5 (dialysed (●) and mixed (○)) at R=4. PicoGreen® solution was added to 2µg/ml pDNA/ of DC-cholesterol lipoplexes, and the increase of PicoGreen® fluorescence was monitored over time at pH 7.4 as described in the Materials and Methods section *2.2.10*. Data were shown as mean +/- standard deviation of three independent experiments.

From these data it can be concluded that the releasing activity of a given anionic lipid depends on the nature of cationic component of the lipoplexes and might be the mesomorphic phases of the anionic lipid used to trigger pDNA release. This will be discussed in detail in chapter 4 in combination with the cryo-TEM experiments.

In accordance to our data, similar studies showed that oleic acid was a very potent pDNA releaser in the case of EDOPC lipoplex, while DOPS showed a lesser efficiency (Tarahovsky et al., 2004). In comparison, pDNA release data from DOTAP/pDNA and DC-cholesterol/pDNA lipoplexes revealed a considerable difference in pDNA releasing capabilities of DOPS and OA containing liposomes. The effect was better observable in DC-cholesterol/pDNA lipoplexes when OA was used. A possible explanation is that the efficiency of anionic lipids to release pDNA could be observed only in terms of the stability of the formed ion-pair between anionic and cationic lipids. This could be partly dependent on the mesomorphic phases and the negative to positive ratio which could form ion-pair at electroneutrality (Tarahovsky et al., 2004; Koynova and MacDonald, 2007).

The dialysed lipoplexes showed low pDNA release under all conditions tested. This was prominent in all dialysed lipoplexes regardless to the type of cationic lipid and N/P ratios used to condense pDNA. The effect of OGP dialysis technique would be suggested to modify the pDNA binding to the cationic lipids.

Based on literature, we assume that the presence of OGP might correlate with the enhancement of charge neutralization of the pDNA phosphate groups. The strong compaction effect of cationic lipid on the pDNA in presence of OGP could be explained by the following expectation:

The non-ionic OGP detergent forms mixed micelles with cationic lipids (Reimer et al., 1995; Gregoriadis 2007). The formed OGP/cationic lipid mixed micelles might have an effect on the distribution of the condensed counter ions surrounding the pDNA (Manning, 1978; Taira et al., 2005) as well as on the water hydration shell (Hackl and Blagoi, 2004). Under these conditions, the interaction between pDNA and cationic lipids is more cooperative (Fig. 3.7). Upon detergent removal, pDNA condensation is promoted which could be facilitated through intra-molecular acyl chain interactions (Reimer et al., 1995).

In addition, it was suggested that the interaction between pDNA and cationic lipids in the presence of OGP is a consequence of electrostatic and hydrophobic interactions (Wong et al., 1996; Zhang et al., 1997; Wasan et al., 1999).

This correlated with our LDA measurements showing a marked reduction in the zeta potential of dialysed lipoplexes compared to mixed lipoplexes. It could be suggested that excess cationic lipids are involved in neutralization of pDNA.

In addition, the effect of OGP concentration on the zeta potential values of pDNA was recorded. Interestingly, the zeta potential values of pDNA were observed to be increased as the OGP concentration in solution is increased (Fig. 3.8). This reflects that the electric double layer disturbance around pDNA might be accompanied by a reduction in the zeta potential negative value of pDNA.

Conclusively, the stronger condensation of pDNA and lower release of pDNA from the lipoplexes as observed for dialysed complexes may also contribute to the low transfection efficiency in previous work (Wheeler et al., 1999; Tam et al., 2000).

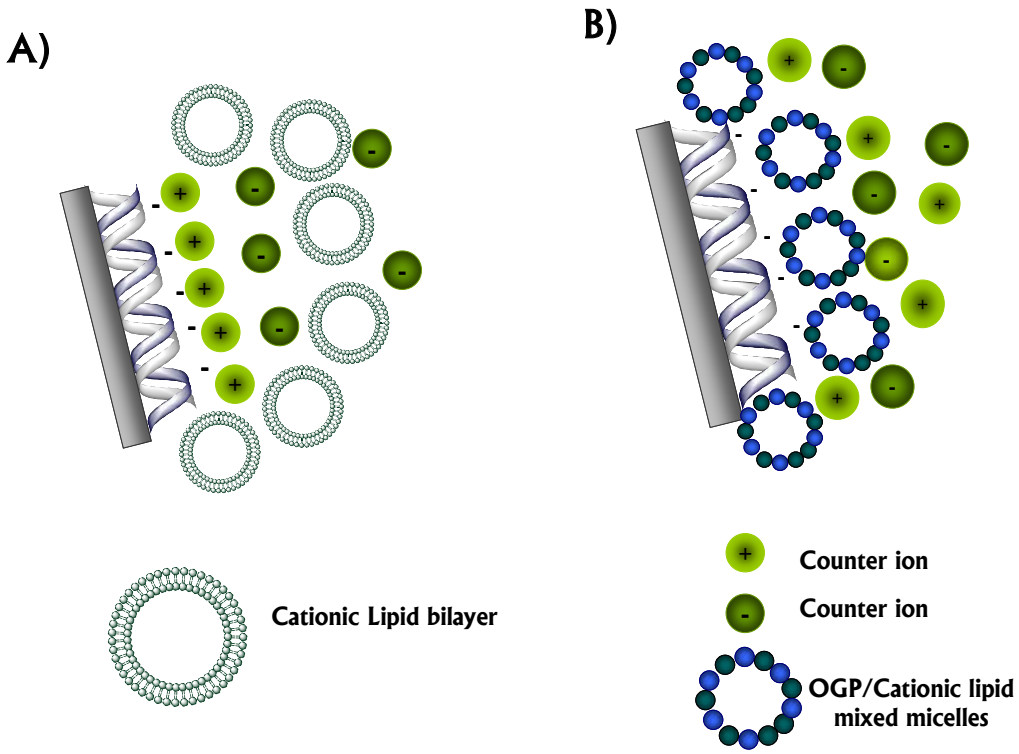

Figure 3.7: Strategies in Cationic lipid/pDNA Lipoplex Preparation. The Lipoplexes is prepared by simple mixing approach (A). An alternative approach, where the pDNA is mixed with cationic liposomes in presence of non-ionic OGP detergent (B). Here a cationic lipid/OGP mixed micelles are formed and should efficiently condense DNA into lipoplexes upon detergent substitution using dialysis technique. This forms aggregated lipoplexes characterised by lower zeta potentials.

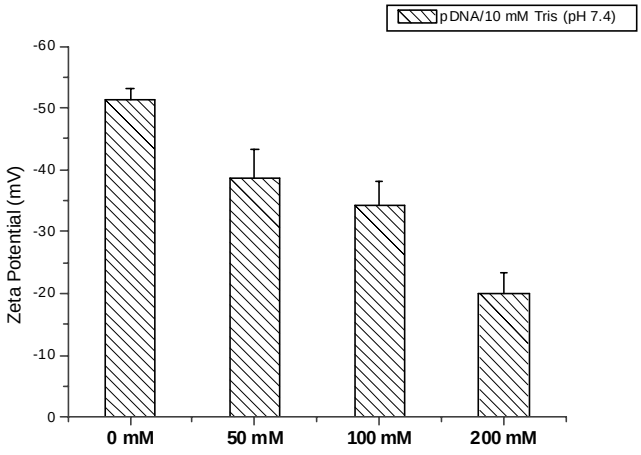

Figure 3.8: Effect of OGP concentration (0-200 mM) on the zeta potential of pDNA. The zeta potential of pDNA was measured by laser Doppler anemometry in three independent experiments in 10 mM Tris, pH 7.4. Results are shown as mean +/- standard deviation.

3.5. pDNA Release from DOTAP/pDNA and DC-cholesterol/pDNA Lipoplexes by DOPS and Oleic Acid (OA) Containing Liposomes at Acidic pH

Rapid release of lipoplexes from the endosomal compartment is supposed to constitute one of the critical steps in determining the efficiency of transfection (Elouahabi and Ruysschaert, 2005; Hoekstra et al., 2007; Caracciolo et al., 2009).

Therefore, we studied the effect of the acidic endosomal pH on the pDNA release from different lipoplexes.

3.5.1. DOTAP/pDNA Lipoplexes

The effect of the acidic pH on the pDNA release from DOTAP/pDNA as well as DC-cholesterol/pDNA lipoplexes at N/P ratio 4 and 5 was examined. Negatively charged liposomes DOPS/DOPE/DOPC (1:1:2) and OA/DOPE/DOPC (1:1:2) were used to initiate pDNA release at R= 4.

In this experiment we observed that DOTAP/pDNA lipoplexes dissociation measured in terms of pDNA release was increased markedly at acidic pH after addition of DOPS/DOPE/DOPC liposomes. As seen in Figure 3.9, a high pDNA release from mixed DOTAP/pDNA lipoplexes at pH 5.3 exhibiting 80 ± 0.46 % at N/P 5 compared to pDNA released at pH 7.4 (40.5 ± 0.7 %). DOTAP/pDNA lipoplexes at N/P 4 revealed a complete DNA release (Fig. 3.10).

We assume that the effect of high H^+ concentration was accompanied by lipoplexes/DOPS liposomes aggregation which induced lipoplexes destabilization and hence, higher pDNA release.

A previous report showed that DOPS containing liposomes exhibited massive non leaky vesicle aggregations in presence of Ca^{+2}. These vesicle aggregations showed a faster overall fusion reaction at high H^+ concentration which is therefore due to the higher aggregation rate rather than the fusion rate per se (Nir et al., 1982).

In contrast, dialysed DOTAP/pDNA lipoplexes showed 3.5 ± 0.5 % and 13.6 ± 0.7 % at N/P 5 and 4 using the same negatively charged liposomes (Fig. 3.9 and Fig. 3.10), respectively. Dialysed DOTAP/pDNA lipoplexes release data confirm highly bound pDNA as reflected by the low DNA release at different pHs.

In contrast, oleic acid efficiency to exert pDNA release from DOTAP/pDNA lipoplexes was not affected by pH change at N/P 5.

OA/DOPE/DOPC liposomes showed 42.3 ± 0.4 % and 46.2 ± 2.9 % pDNA release at N/P 4 and 5, respectively (Fig 3.10 and Fig 3.11 A) when pH decreased to an acidic value.

3.5.2. DC-cholesterol/pDNA Lipoplexes

DC-cholesterol/pDNA lipoplexes treated with DOPS/DOPE/DOPC liposomes showed, in contrast to DOTAP/pDNA lipoplexes, no marked increase in pDNA release (Fig. 3.9 A) compared to pDNA

released at pH 7.4 (Fig. 3.9 B). The protonation state of tertiary amine group of DC-cholesterol lipid at low pH might minimize the ion-pair formation with anionic DOPS lipid.

At N/P 5, mixed DC-cholesterol/pDNA lipoplexes showed 33.7 ± 2.3 % pDNA release which, in contrast to N/P 4 showed only 18.4 ± 0.8 % pDNA release (Fig. 3.9 A and Fig 3.12).

The pDNA release from the DC-cholesterol lipoplexes revealed no correlation between the extent of pDNA release and H^+ concentration after addition of oleic acid containing liposomes. This marked reduction of pDNA release from DC-cholesterol/pDNA lipoplexes at lower N/P ratio verifies that the amount of free tertiary amine group is protonated the condition suggests higher stability of DC-cholesterol/pDNA lipoplexes at acidic pH. From another point of view, the observed reduction in pDNA release might result from the binding capability of pDNA to tertiary DC-cholesterol when pH decreased.

The dialysed DC-cholesterol/pDNA lipoplexes treated with OA/DOPE/DOPC liposomes showed similar behaviour in terms of lower pDNA release.

At pH 5.3, N/P 5 the DC-cholesterol/pDNA lipoplexes exhibited 41.29 ± 0.65 % pDNA release by OA/DOPE/DOPC liposomes which was as equal as pDNA released at neutral pH. Nevertheless, the rate of pDNA release was considerably different showing slower rate in case of acidic pH (Fig. 3.11 A and Fig. 3.11 B). The effect of OA on the mixed DC-cholesterol/pDNA lipoplexes at N/P 4 showed a decrease in pDNA release of 8.84 ± 0.81 % (Fig. 3.12).

Under acidic conditions, DC-cholesterol protonates and the ability to dissociate pDNA both by DOPS/DOPE/DOPC and OA/DOPE/DOPC liposomes, especially at low N/P ratio is reduced.

The inherent buffering capacity of cationic polyamine lipids which have protonizable amines such as DC-cholesterol may take advantage of the “proton-sponge” effect. This effect promotes a continual influx of ions and water, causing a disruption of the vesicle (Rolland and Sullivan, 2003), for more details see page 21.

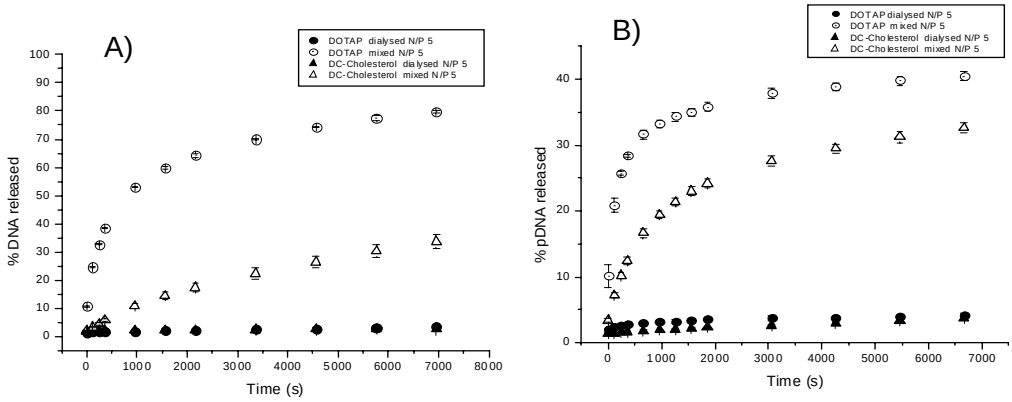

Figure 3.9: pH-dependent effect on the pDNA release from DOTAP/pDNA and DC-cholesterol/pDNA lipoplexes at N/P 5 using DOPS/DOPE/DOPC (1:1:2) liposomes. DOTAP/pDNA (dialysed (●) and mixed (○)) and DC-cholesterol/pDNA (dialysed (▲); mixed (Δ)) lipoplexes were treated with DOPS/DOPE/DOPC (1:1:2) at pH 5.3 (A), and 7.3 (B) at R=4. PicoGreen® solution was added to 2µg/ml pDNA/ of DOTAP or DC-cholesterol lipoplexes, and the increase of PicoGreen® fluorescence monitored over time at pH 7.4 as described in the Materials and Methods section *2.2.10*. Data were shown as mean +/- standard deviation of three independent experiments.

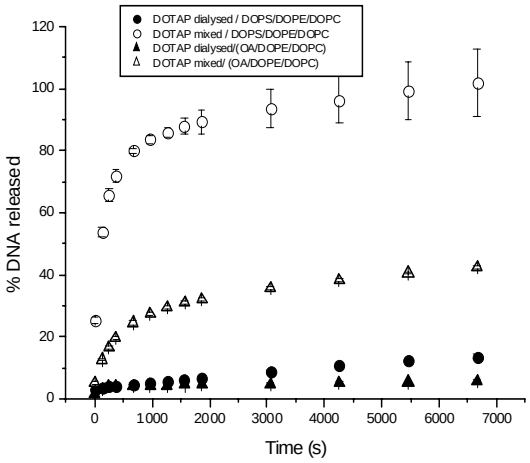

Figure 3.10: pH-dependent effect on the pDNA release from DOTAP/pDNA lipoplexes at N/P 4 using various anionic liposomes. DOTAP/pDNA lipoplexes were treated with OA/DOPE/DOPC (1:1:2) (dialysed (▲); mixed (Δ)) and DOPS/DOPE/DOPC (1:1:2) (dialysed (●) and mixed (○)) liposomes at pH 5.3 at R=4. PicoGreen® solution was added to 2µg/ml pDNA/ of DOTAP lipoplexes, and the increase of PicoGreen® fluorescence monitored over time at pH 7.4 as described in the Materials and Methods section 2.2.10. Data were shown as mean +/- standard deviation of three independent experiments.

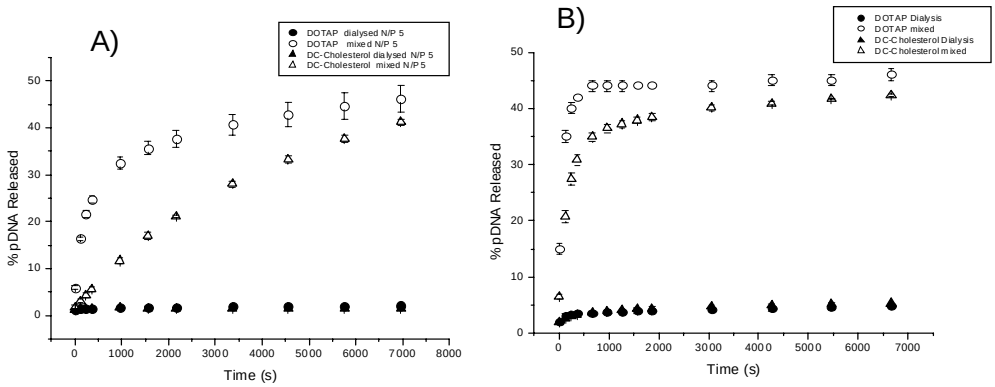

Figure 3.11: pH-dependent effect on the pDNA release from DOTAP/pDNA and DC-cholesterol/pDNA lipoplexes at N/P 5 using OA/DOPE/DOPC (1:1:2) liposomes. DOTAP/pDNA (dialysed (●); mixed (○)) and DC-cholesterol/pDNA lipoplexes (dialysed (▲) and mixed (Δ)) were treated with OA/DOPE/DOPC liposomes at pH 5.3 (A) and pH 7.3 (B) at R=4. PicoGreen® solution was added to 2µg/ml pDNA/ of DOTAP or DC-cholesterol lipoplexes, and the increase of PicoGreen® fluorescence monitored over time at pH 7.4 as described in the Materials and Methods section *2.2.10*. Data were shown as mean +/- standard deviation of three independent experiments.

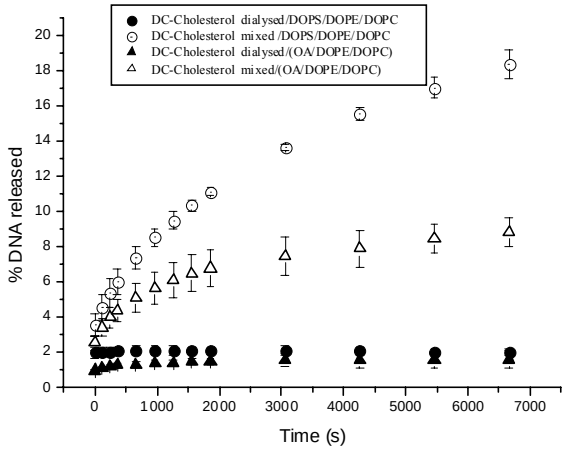

Figure 3.12: pH-dependent effect on the pDNA release from DC-cholesterol/pDNA lipoplexes at N/P 4 using various anionic liposomes. DC-cholesterol /pDNA were treated with OA/DOPE/DOPC (1:1:2) (dialysed (▲); mixed (Δ)) and DOPS/DOPE/DOPC (1:1:2) (dialysed (●) and mixed (○)) liposomes at pH 5.3 and R=4. PicoGreen® solution was added to 2µg/ml pDNA/ of DC-cholesterol lipoplexes, and the increase of PicoGreen® fluorescence monitored over time at pH 7.4 as described in the Materials and Methods section *2.2.10*. Data were shown as mean +/- standard deviation of three independent experiments.

3.6. Conclusion

Here, we show that the superior stability of dialysed lipoplexes does correlate with their limited capability to release DNA by different negatively charged liposomes both at pH 7.4 and 5.3. Our results may be potentially important for the development of new strategies toward the rational design of lipoplexes by enhancing their destabilization ability.

The effect of OGP detergent dialysis as an alternative technique to produce lipid-based pDNA influenced the physicochemical properties of the produced lipoplexes. This was clearly seen by a marked reduction in the zeta potential values upon detergent dialysis. As a consequence, the colloidal stability of the lipoplex decreased showed tendency to aggregate. This was observed by large hydrodynamic diameter compared to mixed lipoplexes.

The presence of OGP during cationic lipid/pDNA interaction enables cooperative interaction of the cationic lipid with pDNA. We assume that the effect of OGP may alter the counter-ion condensed layer which considered as a barrier between cationic compounds to neutralize sufficient negative phosphates carried by pDNA.

Interestingly, in absence of OGP during lipoplex assembly, pDNA release from lipoplexes was pronounced when different negatively charged liposomes were applied.

It seems that the non-ionic detergent can play a strong protective role for the pDNA. We will therefore further examine the morphological features of the dialysed lipoplexes to visualize the compaction effect of the cationic DOTAP lipid on pDNA in the DOTAP/pDNA lipoplexes.

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CHAPTER 4

Cryo-TEM Visualization of DOTAP/pDNA Lipoplexes Prepared by Detergent Dialysis Technique

Abstract

Previously we reported on the pDNA condensation state in DOTAP/pDNA lipoplexes prepared by detergent N-octyl-beta-D-glucopyranosid (OGP) dialysis approach compared to mixed lipoplexes. pDNA release using PicoGreen® assay as a nucleic acid marker was estimated when anionic lipids DOPS and oleic acid (OA) containing liposomes were initiated pDNA release from the lipoplexes. pDNA release was considered negligible from dialysed lipoplexes as compared to mixed DOTAP/pDNA lipoplexes. Nevertheless, negatively charged liposomes of OA/DOPE/DOPC (1:1:2) exhibited more efficient pDNA release capabilities than DOPS/DOPE/DOPC (1:1:2) in terms of rate and extent of pDNA release.

In the present study, DOTAP/pDNA lipoplexes were formed by an alternative technique than simple mixing process complexing pDNA and DOTAP in the presence of 200 mM non-ionic detergent N-octyl-beta-D-glucopyranosid (OGP) at N/P ratio 5. After 30 min incubation, the detergent substitution was performed using membrane dialysis. The mixed and dialysed lipoplexes were characterized using cyro-TEM to visualize the influence of OGP on the morphological features of assembled DOTAP/pDNA lipoplexes.

The mechanisms by which pDNA released from DOTAP/pDNA lipoplexes using different anionic lipids were visualized.

The preparation technique using detergent OGP dialysis influenced the morphological characteristics of the DOTAP/pDNA lipoplexes. Cryo-TEM revealed that dialysed DOTAP/pDNA lipoplexes formed compact aggregated structures of pDNA than that observed in mixed DOTAP/pDNA lipoplexes.

This correlated well with pDNA condensation using PicoGreen® assay characterised by lower pDNA de-condensation capability from dialysed lipoplexes.

The mechanism of pDNA release by DOPS/DOPE/DOPC (1:1:2) liposomes was different than by OA/DOPE/DOPC liposomes. This was observed for both mixed and dialysed DOTAP/pDNA lipoplexes.

DOPS/DOPE/DOPC liposomes induced pDNA release by vesicle adhesion characterised by massive aggregation between negatively charged liposomes and cationic DOTAP lipoplexes. While, OA/DOPE/DOPC liposomes induced pDNA release by “inverted” non-lamellar intermediates formation.

In conclusion, the preparation technique influenced the morphological features of the produced lipoplexes. Two different mechanisms were observed to induce pDNA release from DOTAP/pDNA lipoplexes. Regardless to the method of preparation, both mechanisms were observed in mixed and dialysed lipoplexes. Yet the effect of anionic/ cationic lipid phase formation during pDNA release was more pronounced in mixed than dialysed DOTAP/pDNA lipoplexes.

4.1. Morphology of DOTAP/pDNA Lipoplexes

As a first experiment, before the interaction with anionic liposomes was investigated, the morphology of pure DOTAP/pDNA lipoplexes was characterized using cryo-TEM. The interaction between plasmid DNA and the cationic lipid in bulk phase is a very fast process which is governed by electrostatic interactions between the phosphate group of the pDNA and the cationic head groups of the cationic amphiphile.

Gershon et al., (1993) have proposed a model for cationic liposome/pDNA complexation. At low ratios of liposomes to pDNA, positive vesicles are adsorbed to pDNA to form aggregates that gradually surround larger segments of the pDNA. As the amount of liposomes is increased, the aggregated liposomes along the pDNA reach critical concentrations and charge densities at which membrane fusion and pDNA collapse processes are initiated. Upon additional increase of the liposome concentration, the collapsed pDNA structures are covered completely by the lipid bilayers. The size of the final product is dependent on the size of the initial lipid vesicles, the nitrogen/phosphate ratio and ionic strength of the medium. pDNA condensation is caused by mechanisms independent of the length of the individual pDNA molecules (Reich et al., 1991; Bloomfield, 1991) and the absolute concentration of the nucleic acids (Gershon et al., 1993). These complexes form clusters of lipid molecules and not a vesicularized membrane bilayer, and therefore lack an internal aqueous space.

4.1.1 Visualization of Mixed DOTAP/pDNA Lipoplexes

Mixed lipoplexes of DOTAP/pDNA were prepared by mixing technique at N/P ratio 5 as described in chapter 2 the Materials and Method section 2.2.2.1. Structural details of the mixed DOTAP/pDNA lipoplexes were visualized by cryo-TEM (Fig 4.1).

The addition of pDNA to preformed cationic liposomes triggers significant structural changes in the liposomes as well as the pDNA. Pure cationic DOTAP liposomes were mostly unilamellar vesicles in the range between 80 and 100 nm diameters. After pDNA contact with cationic lipid vesicles, a marked fusion of the cationic vesicles was observed. This could be explained by wrapping pDNA around cationic DOTAP vesicles. More cationic vesicles adhered to the pDNA wrapped vesicle were also observed (Fig. 4.1 A and B). Addition of pDNA to lipid caused aggregation of most vesicles into larger complexes. The size distribution shown in cyro-TEM pictures indicated vesicles of heterogeneous distribution in size of more than 200 nm. This correlated to our PCS measurements shown in chapter 3.

Eventually rupture and collapse of vesicles occurred. These events of adhesion and fusion indicate that the vesicles had acquired a coating of pDNA. Otherwise cationic vesicles exhibited no interactions with each other.

Figures 4.1 C and D show DOTAP vesicles wrapped by pDNA where two initially separate membranes forced into close contact by bound pDNA showed a thick border compared to pure

DOTAP vesicles. In some lipoplexes (Figure 4.1 B), the inter bilayer binding is discontinuous and the outer membrane exhibits a bulb-like form.

These consecutive adhesions led to eventual collapse of more than one DOTAP vesicle wrapped pDNA aggregates (Fig 4.1 E).

Different membrane morphology was also observed by Xu et al., (1999) showing that DOTAP/pDNA lipoplexes had many spike-like strands similar to our observation in Figure 4.1 E. In accordance, Lasic, (1997) observed that DOTAP/pDNA lipoplex form loose aggregations without DNA/lipid fibers.

In conclusion, the mixed lipoplexes were found to show structures according to the proposed model of pDNA/cationic lipid complexation (Felgner et al., 1987; Felgner and Ringold, 1989; Gershon et al., 1993; Lasic and Templeton, 1996; Xu et al., 1999).

4.1.2 Visualization of Dialysed DOTAP /pDNA Lipoplex

Dialysed lipoplexes of pDNA and DOTAP lipid were prepared by detergent OGP dialysis technique. Dialysed lipoplexes were prepared in presence of 200 mM OGP at N/P ratio 5 followed by dialysis as described in chapter 2 the Materials and Method section 2.2.2.2.

Structural details of the dialysed DOTAP/pDNA lipoplexes were visualized by cryo-TEM. Dialysed DOTAP/pDNA lipoplexes showed highly condensed particles (Fig 4.2 A). These collapsed structures indicate that the compaction effect of DOTAP lipid on pDNA is enhanced during lipoplex assembly. For example, a detailed figure (Figure 4.2 B) showed a triplet structure consisting of a dark central line surrounded on both sides by a bright band alternating with a dark line, which could be explained as two cationic bilayer membranes adsorbed to one other. The contact between the bilayers suggested that this higher contrast resulted from the presence of pDNA sandwiched between the membranes. Another indication for pDNA location is the fact that the membranes are not electrostatically repelled from each other which would be expected to cause large aggregates.

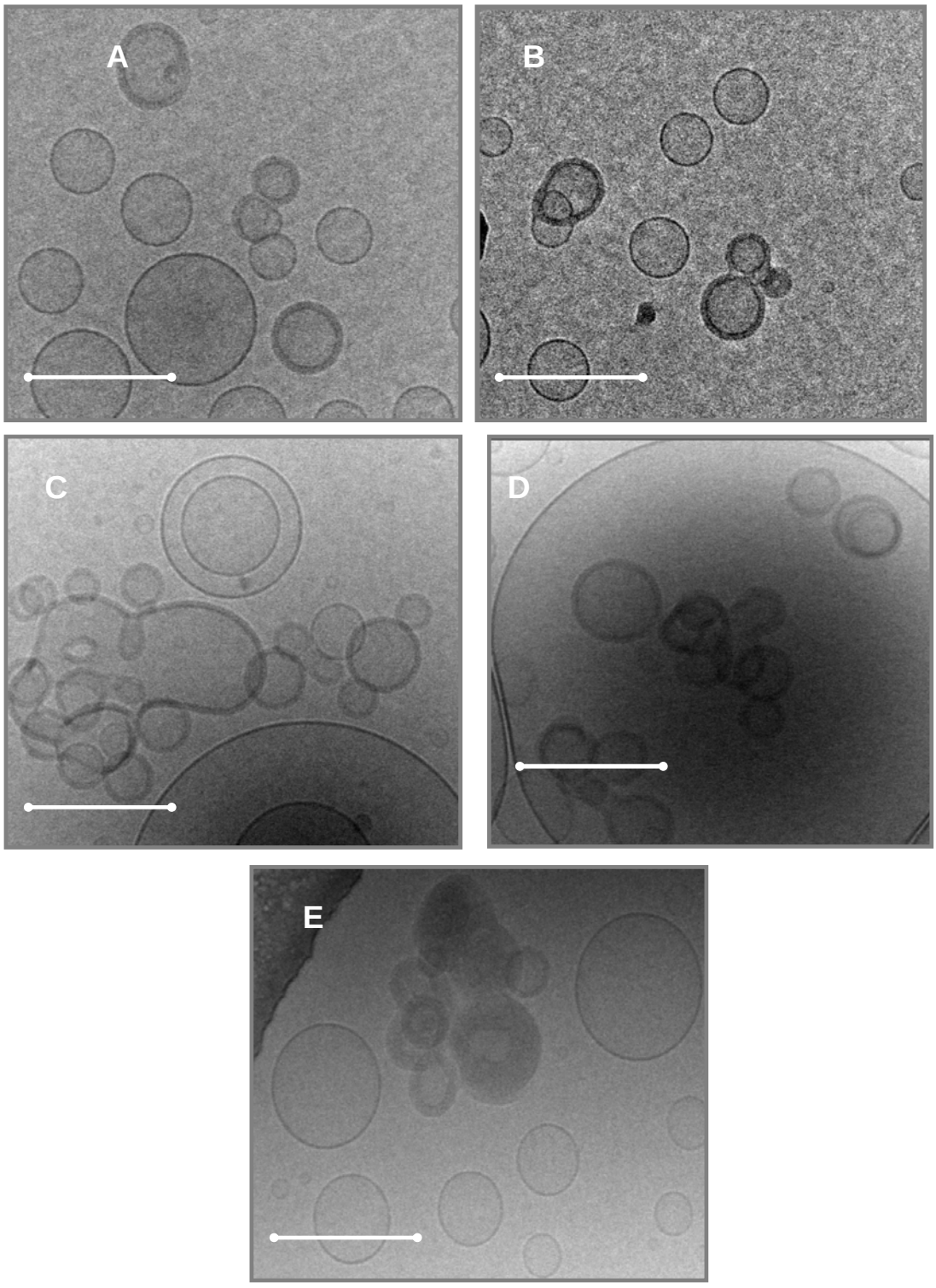

Figure 4.1: Cryo-TEM micrographs of mixed DOTAP/pDNA lipoplexes. The mixed DOTAP/pDNA lipoplexes at N/P 5 were prepared and visualized by cryo-TEM as described in the Materials and Methods section *2.2.12.* The figure shows complexes of DOTAP/pDNA prepared by mixing technique in 145 mM NaCl/10 mM HEPES, pH 7.4. Bars are 200 nm.

The aggregates according to cyro-TEM photographs showed large sized lipoplexes of approx. > 800 nm. In correlation to PCS measurements, a marked increase in the particle size was clearly observed for dialysed DOTAP/pDNA lipoplexes compared to mixed lipoplexes. The hydrodynamic diameter measured by PCS showed an increase from 297.5± 20.22 nm (mixed) to 601.3 ± 311.13 nm (dialysed).

In contrast, in the presence of the OGP detergent the driving force for mixed micelles self-assembly is not cohesion between the hydrocarbon chains, but the so-called hydrophobic effect (Widom et al., 2003). The hydrophobic effect responsible for the self assembly is in principle an entropically driven effect arising from the hydrogen bonded structures of water. In the pure water phase, strong hydrogen bonds between water molecules are formed. When a hydrocarbon molecule is added to the water phase, the water/water bonds have to be disrupted.

In presence of OGP, it was assumed that these water/water bonds could be disrupted as previously described (Gregoriadis, 2007). We assume that the effect of detergent could induce cooperative contact between cationic lipid and negatively charged phosphates of pDNA which may provoke pDNA condensation upon detergent dialysis.

It was demonstrated that pDNA lipid particles obtained by the detergent dialysis technique is protected from the external environment. As marker for protection, pDNA was completely protected against nuclease digestion (Hofland et al., 1996; Wheeler et al., 1999). In conclusion, the ultra-structure of the dialysed lipoplexes showed an ordered and high compaction level of pDNA which leads to more stable lipoplexes compared to mixed cationic lipid/pDNA systems.

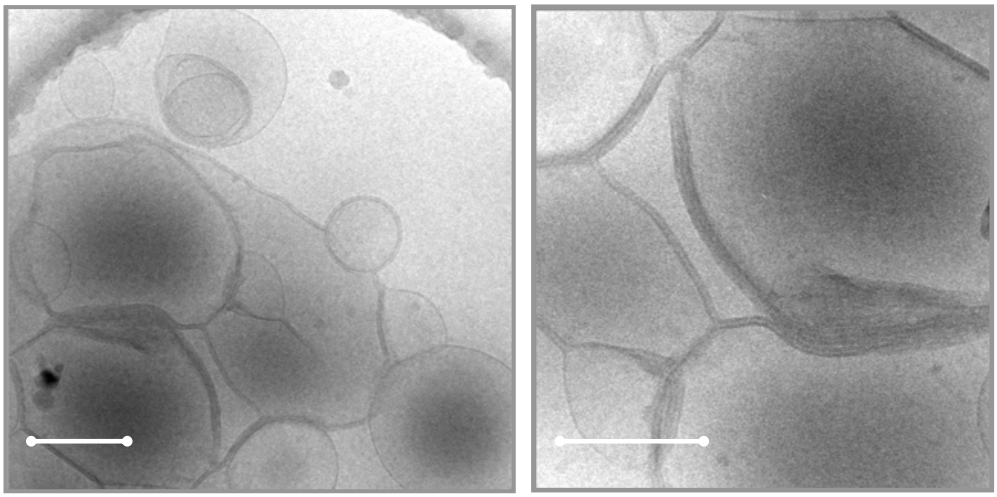

Figure 4.2: Cryo-TEM micrographs of dialysed DOTAP/pDNA lipoplexes. The dialysed DOTAP/pDNA lipoplexes at N/P 5 were prepared and investigated by cryo-EM as described in the Materials and Methods section *2.2.12*. In A, complexes consisting of DOTAP/pDNA prepared by detergent dialysis technique in 20 mM HEPES/145 mM NaCl, pH 7.4 are shown. In B an enlarged micrograph of A showed huge DOTAP/pDNA lipoplexes which appeared as a collapsed structure. Bars are 200 nm.

4.2. Visualization of Interaction of DOTAP/ pDNA Lipoplexes with Different Negatively Charged Liposomes

In this study, we performed cryo-TEM experiments and investigated the mesomorphic structures developing during the pDNA release when anionic lipids were added.

DOTAP/pDNA lipoplexes were prepared by mixing and detergent dialysis techniques at N/P 5, and the negatively charged DOPS/DOPE/DOPC (1:1:2) and OA/DOPE/DOPC (1:1:2) liposomes at $R = 4$, where $R = A/Lc$ (moles of anionic lipid/moles of cationic lipid) were used.

Cyro-TEM micrographs immediately and 1 hour after incubation were visualized. The morphological changes were visualized as described in chapter 2, the Materials and Method section *2.2.12*.

4.2.1. Visualization of DOTAP/pDNA Lipoplexes after Interaction with DOPS Containing Liposomes

4.2.1.1. Interaction of Mixed DOTAP/pDNA Lipoplexes with DOPS Containing Liposomes

Cryo-TEM micrographs of pure negatively charged DOPS/DOPE/DOPC (1:1:2) liposomes revealed spherical, unilamellar lipid bilayer vesicles (Fig. 4.3). The anionic liposomes were monodisperse distributed vesicles and showed diameter of approx. 80 nm.

DOPS containing liposomes showed massive aggregations when incubated with mixed DOTAP/pDNA lipoplexes governed mainly by electrostatic interactions between DOTAP/pDNA lipoplexes and anionic liposomes. Aggregations were accompanied by mutual flattening of the anionic liposomes. When compared to the pure anionic liposomes, a marked increase in size of anionic liposomes was observed. Furthermore, adhesion was characterized by the formation of thick contact layers where the lipoplex adhered to the anionic liposomes indicating direct molecular contact between the membranes of the opposite charge (Fig 4.4). The observed thick contact layer is an evidence for the collapse of lipoplexes to many adjacent DOPS liposomes, becoming immediately apparent as the lipoplex and anionic surfaces came into contact.

To recapitulate our cryo-TEM observations, several features of mixed DOTAP/pDNA lipoplexes and DOPS/DOPE/DOPC liposomes were observed:

I. Rupture of the anionic vesicle could not be observed.

II. Adhesion of lipoplexes to DOPS containing liposomes, which showed size expansion in all cyro-TEM fields. The increase in size was 5-10 times the size of the original DOPS liposomes.

III. Mixed DOTAP/pDNA lipoplexes remained attached to the anionic vesicle.

IV. Squeezing of DOTAP/pDNA lipoplexes was seen after adhesion to DOPS liposomes leading to a localized collapse.

A similar observation was done by Berezhna et al., (2005) using laser scanning imaging in conjunction with fluorescence cross-correlation analysis. They visualized intermembrane interactions between DOTAP lipoplexes and giant uni-lamellar vesicles (GUVs) composed of DOPS/DOPE/DOPC. They observed a binding of lipoplex at the surface of the GUV, but no rupture of the vesicles was detected. Importantly, this might be indicative of lipid translocation to the outer leaflet of the bilayer (Kahya et al., 2002), as has been proposed to occur for DOPS translocation from outer to inner leaflet within the endosomal compartment (Xu and Szoka, 1996). During lipoplex/GUV interaction, polynucleotides with a length of 66 base pairs were effectively delivered inside the GUV, which was enhanced when the GUV contained increasing levels of DOPS and DOPE, by charge neutralization of the cationic lipid.

In this study, the morphological changes of DOPS liposomes and DOTAP/pDNA lipoplexes mixture were also monitored after longer incubation time of 1 hour. Subsequent events generally led to changes in vesicle membrane integrity (Fig. 4.5) were observed. Lipid tubular like forms was also found to observe, as indicated by arrows (Fig. 4.5 B and Fig. 4.5 C).

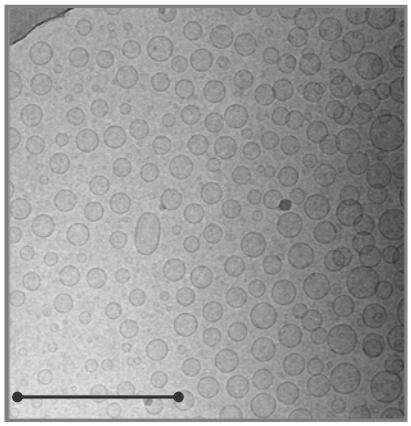

Figure 4.3: Cryo-TEM micrograph of pure negatively charged DOPS/DOPE/DOPC (1:1:2). Liposomes were visualized by cryo-EM before addition to lipoplexes. Bar is 500 nm.

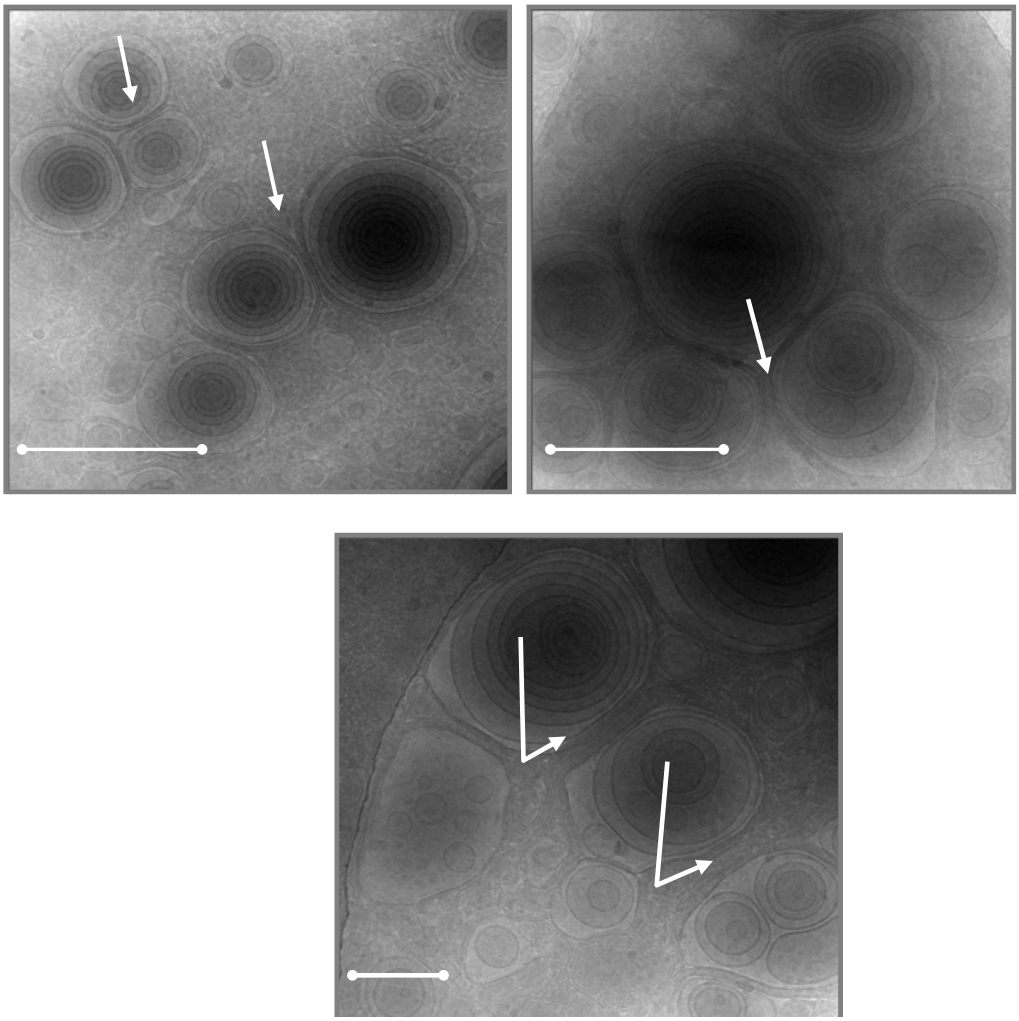

Figure 4.4: Cryo-TEM micrographs of mixed DOTAP/pDNA lipoplexes after interaction with DOPS containing liposomes. Interactions between DOTAP/pDNA lipoplexes and anionic liposomes were visualized by cryo-EM immediately after mixing. Anionic vesicles adhesion was induced by electrostatic attraction forces. The mixed DOTAP lipoplexes/DOPS containing liposomes were investigated by cryo-EM as described in the Materials and Methods section *2.2.12*. Bars are 500 nm.

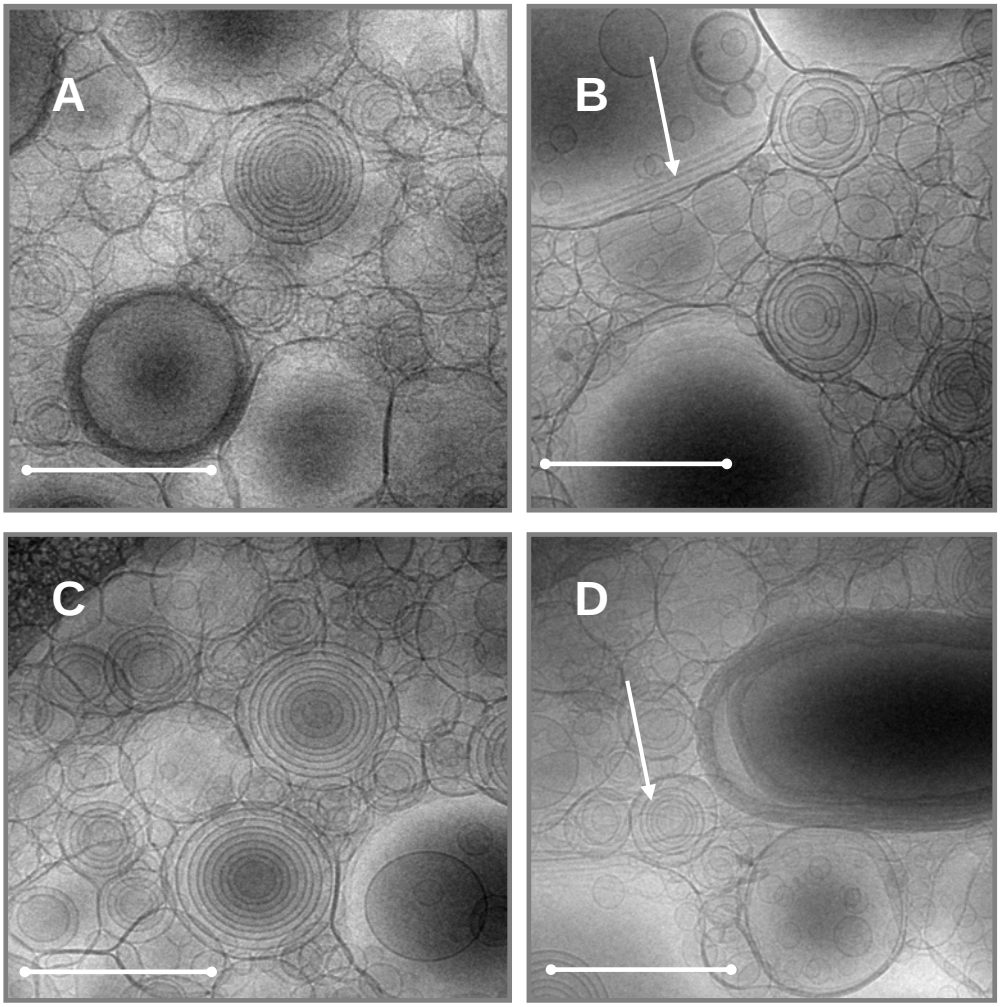

Figure 4.5: Cryo-TEM micrographs of mixed DOTAP/pDNA lipoplexes after interaction with DOPS containing liposomes. Negatively charged liposomes were visualized by cryo-TEM one hour after addition to mixed lipoplexes. The mixed DOTAP lipoplexes/DOPS containing liposomes were investigated by cryo-EM as described in the Materials and Methods section *2.2.12*. Bars are 500 nm.

After longer contact, we have observed some vesicles deformation. These morphological changes could be due to the increase in the vesicle membrane tension as previously described (Evans and Parsegian, 1983; Parsegian and Rand, 1983).

Seifert and Lipowsky (1990) also observed that strong adhesive forces between vesicles leads to surface deformation as a consequence of increased edge stress. We suppose that the edge stress eventually induces rupture of the adsorbed vesicles presumably leading to complete pDNA release with simultaneous transformation of vesicle bilayer into planar lipid bilayer and/or neutralized ion pair of DOTAP/DOPS bilayers. This indicates a lipoplex disassembly (Fig. 4.5).

In a previous study, using synchrotron small angle x-ray diffraction (SAXD), Caracciolo et al., (2006) showed that only lamellar lipoplexes were found to exist when phosphatidylglycerol (DOPG), a lamellar bilayer forming lipid, is used to exert pDNA release. In this study, a characteristic structural feature of the DOTAP/DOPC/pDNA lipoplexes and DOPG mixture was the extensive swelling of the lamellar phase of lipoplexes that accompanied its enrichment with lamellar DOPG. Other studies supposed that electrostatic interactions between the cationic lipids of the lipoplex and the anionic phospholipids that laterally diffuse within lipoplex result in simultaneous charge neutralization of cationic lipids and weakening of the cationic lipid/pDNA interaction(Xu and Szoka 1996; Caracciolo et al., 2006).

4.2.1.2. Interaction of Dialysed DOTAP/pDNA Lipoplexes with DOPS Containing Liposomes

As shown for mixed DOTAP/pDNA lipoplexes, a massive surface expansion of negatively charged liposomes upon contact with lipoplexes was a characteristic feature in all cryo-TEM micrographs. The vesicle expansion was indicated by an increase of the vesicle diameter immediately after the reaction was initiated.

The strong compaction of pDNA in dialysed lipoplexes observed in cryo-TEM micrographs was accompanied by reduction in the zeta potential values, according to PCS measurements. The dialysed DOTAP/pDNA lipoplexes showed 18 % reduction in positive zeta potential value at N/P 5 compared to mixed DOTAP/pDNA lipoplexes.

Upon contact of DOPS/DOPE/DOPC liposomes with dialysed DOTAP/pDNA, no thick layer of contact compared to mixed DOTAP/pDNA lipoplexes.

Although a collapse of dialysed lipoplexes was not observed, cryo-TEM micrographs showed similarly lamellar structures as observed with mixed lipoplexes (Fig. 4.6).

In some cyro-TEM fields (Fig. 4.6 A and Fig. 4.6 C), we observed vesicles showing a semifusion contact, as indicated by arrows, including only the outer monolayer membranes merging.

Similar to mixed DOTAP/pDNA lipoplexes after immediate contact with DOPS vesicles, dialysed lipoplexes showed similar aggregates after one hour incubation (Fig. 4.7). This could be interpreted by the effect of DOPS containing liposomes to partially dissociate DOTAP lipid upon longer contact by electrostatic attraction forces. However, after longer incubation, vesicle deformation was not observed in case of dialysed DOTAP/pDNA lipoplexes.

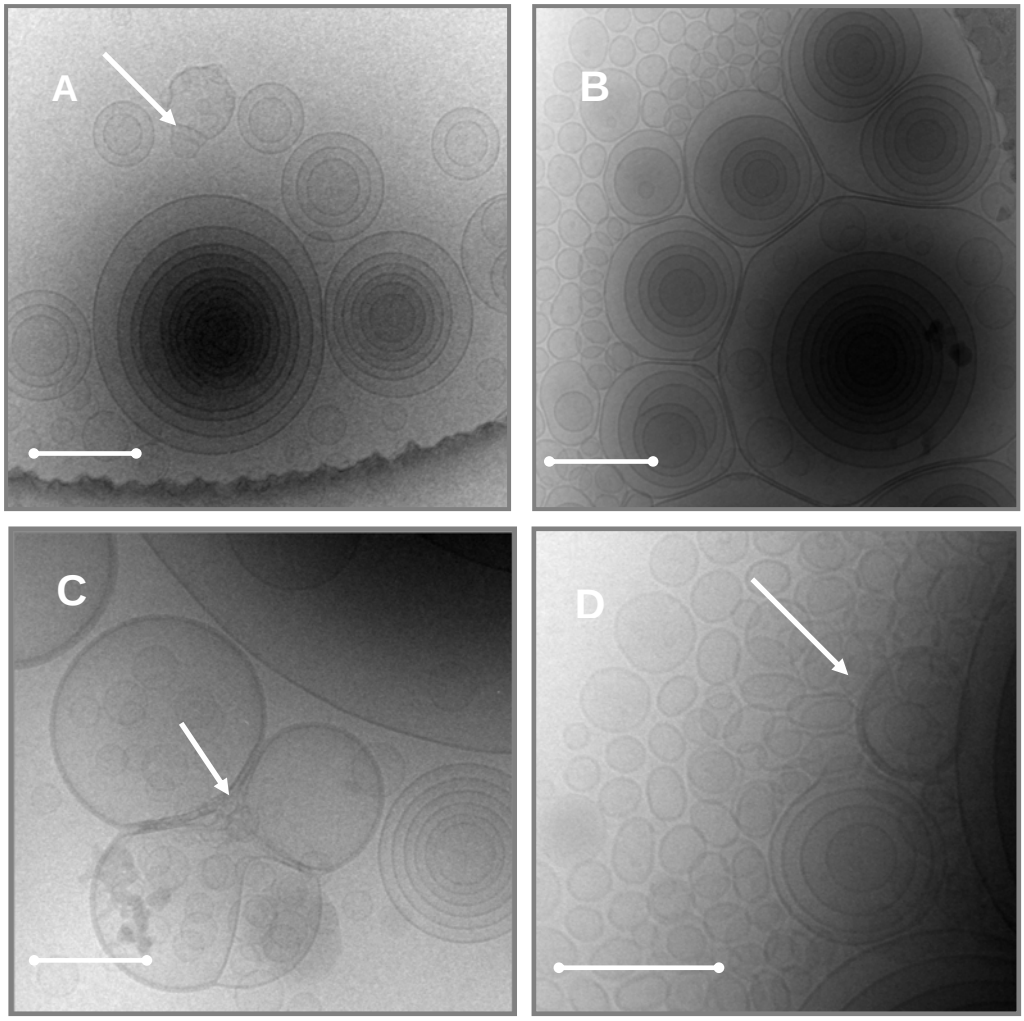

Figure 4.6: Cryo-TEM micrographs of dialysed DOTAP/pDNA lipoplexes after interaction with DOPS containing liposomes. Anionic liposomes were visualized by cryo-TEM immediately after addition to dialysed DOTAP/pDNA lipoplexes. The dialysed DOTAP/pDNA lipoplexes/DOPS containing liposomes were investigated by cryo-EM as described in the Materials and Methods section *2.2.12*. Bars are 200 nm.

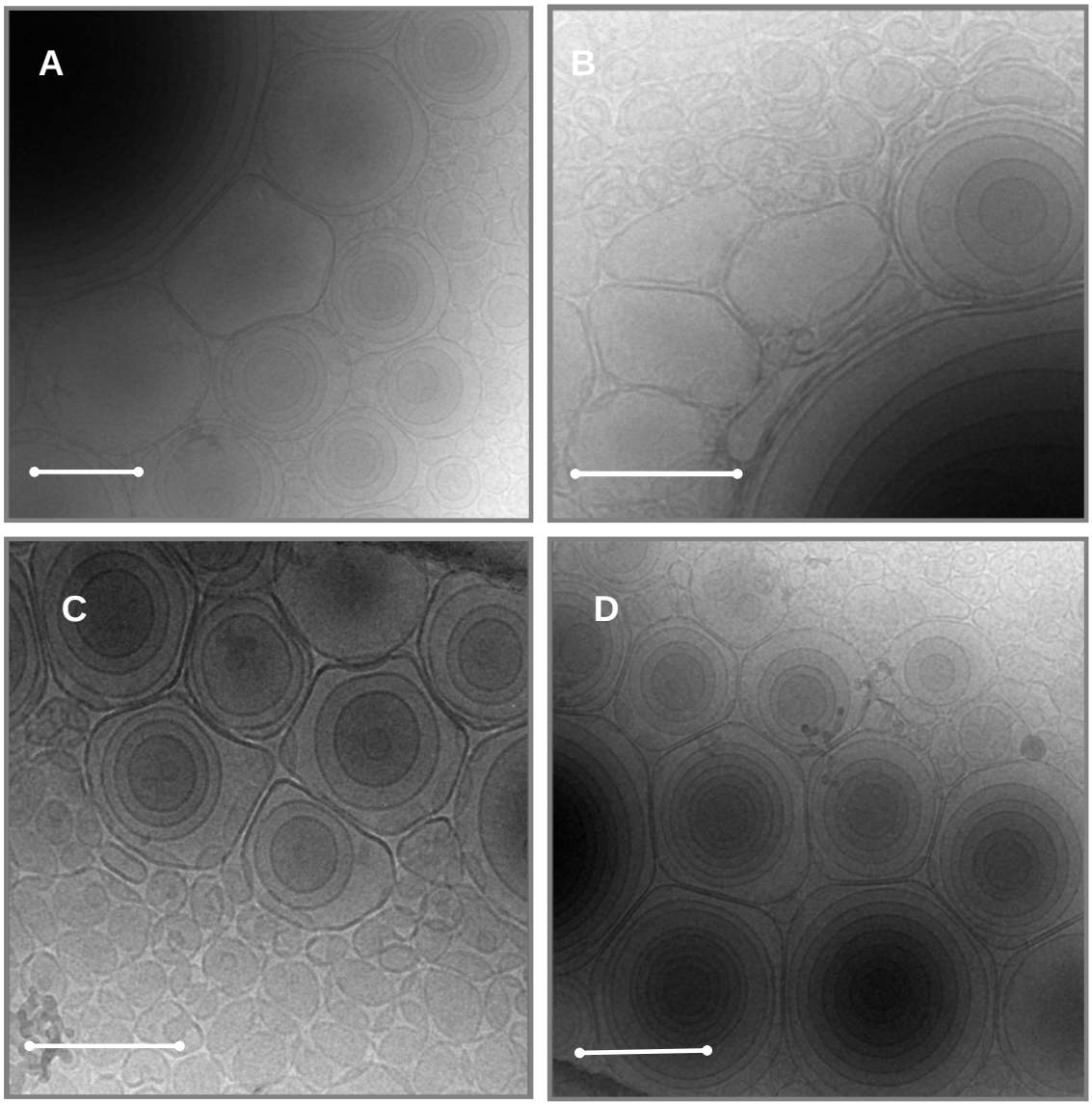

Figure 4.7: Cryo-TEM micrographs of dialysed DOTAP/pDNA lipoplexes after interaction with DOPS containing liposomes Anionic liposomes were visualized by cryo-TEM one hour after addition to dialysed DOTAP/pDNA lipoplexes. The dialysed DOTAP/pDNA lipoplexes/DOPS containing liposomes were investigated by cryo-EM as described in the Materials and Methods section *2.2.12*. Bars are 200 nm.

4.2.2. Visualization of DOTAP/ pDNA Lipoplexes upon Interaction with Oleic Acid Containing Liposomes

From our pDNA release data in chapter 3, oleic acid (OA) in combination with DOPE/DOPC liposomes was found to be more efficient in terms of the release rate of pDNA from DOTAP/pDNA lipoplexes compared to DOPS/DOPE/DOPC liposomes.

We were particularly interested whether different mesomorphic organizations of the DOTAP/OA lipid mixtures might correlate with the pDNA release compared to DOPS containing liposomes described above.

Cryo-TEM study revealed that the structural features of mixed as well as dialysed DOTAP/pDNA lipoplexes were accompanied by the appearance of "inverted" non-bilayer lipid structures as fusion intermediates when oleic acid was used to exert pDNA release.

In more detail, as shown in Figure 4.8, cyro-TEM micrographs showed a characteristic of three dimensionally organized, striated pattern of lipids which organized in "inverted" non-bilayer phases.

Previous studies reported that oleic acid undergoes a lamellar to inverted hexagonal phase transition (Epand et al., 1991; Tarahovsky et al., 2004). We assume that oleic acid/DOTAP ion pair formation is mediated by "inverted" non-bilayer lipid phase separation.

One hour later, a size increase of the "inverted" non-bilayer phase intermediates was observed only in mixed lipoplexes (Fig. 4.9).

We reasoned that the increase in size of DOTAP/OA lipid intermediates due to excess DOTAP lipids disassembled from mixed lipoplexes showed size enlargement of these fusion intermediates in presence of excess OA approaching neutrality.

In line to our observation, Ruthven et al., (2000) showed that the ability to form "inverted" non-lamellar phases is maximal in mixtures where the mean surface charge of membranes approaches neutrality and decreases with the increase in the density of positive or negative charges at the membrane surface.

Vesicle systems that undergo a lamellar to inverted hexagonal $L\alpha$-H_{II} phase transition rapidly develop so called inverted micellar intermediates (IMI) between the bilayers of the contacted vesicles (Figure 4.10). These fusion intermediates are known as lipidic particles (Verkleij et al., 1980; Siegel 1986 a, b) and can either assemble into vesicles or they may evolve into so called interlamellar attachment sites (ILA) formed at the site of the interbilayer contact and this transformation will result in mixing of the bilayer lipids.

The structural organization of dialysed DOTAP lipoplexes was similar to mixed DOTAP lipoplexes in presence of oleic acid liposomes. Nevertheless, in case of dialysed lipoplexes, the "inverted" non-bilayer phase separation showed smaller size of approx. 200 nm diameters than that observed in mixed lipoplexes (Fig. 4.11 A-D).

Unlike mixed lipoplexes, longer incubation time revealed disappearance of lipidic intermediates in case of dialysed lipoplexes (Fig. 4.12 A and Fig. 4.12 B). The transformed intermediate structures

found for dialysed lipoplexes (Figure 4.12) suggested complete lipidic particle dissociation into vesicles composed presumably of neutralized ion-pair vesicles.

This process of phase conversion from lamellar to non-lamellar phase, which may include the hexagonal H_{II} or H_I as well as the micellar cubic phase, also seems to be influential in causing efficient dissociation of the pDNA from the lipoplex (Zuhorn et al., 2005; Koynova et al., 2005; Wasungu et al., 2006; Ewert et al., 2006).

The stability of such phases may possibly depend on the nature of the anionic and cationic lipids involved (Koynova et al., 2005). This was suggested by revealing a highly stable curved micellar cubic phase, induced in lipoplexes consisting of a cationic lipid, upon their interaction with cardiolipin-containing bilayers. At these conditions, the highest pDNA dissociation was obtained which showed a transient formation of the micellar cubic phase.

A situation can be also obtained in lipid mixtures containing DOPS and phosphatidylethanolamine (DOPE), where the presence of Ca^{2+} can allow the preference of DOPE to form H_{II} phase to express (Cullis and Verkleij, 1979; Hope and Cullis, 1979).

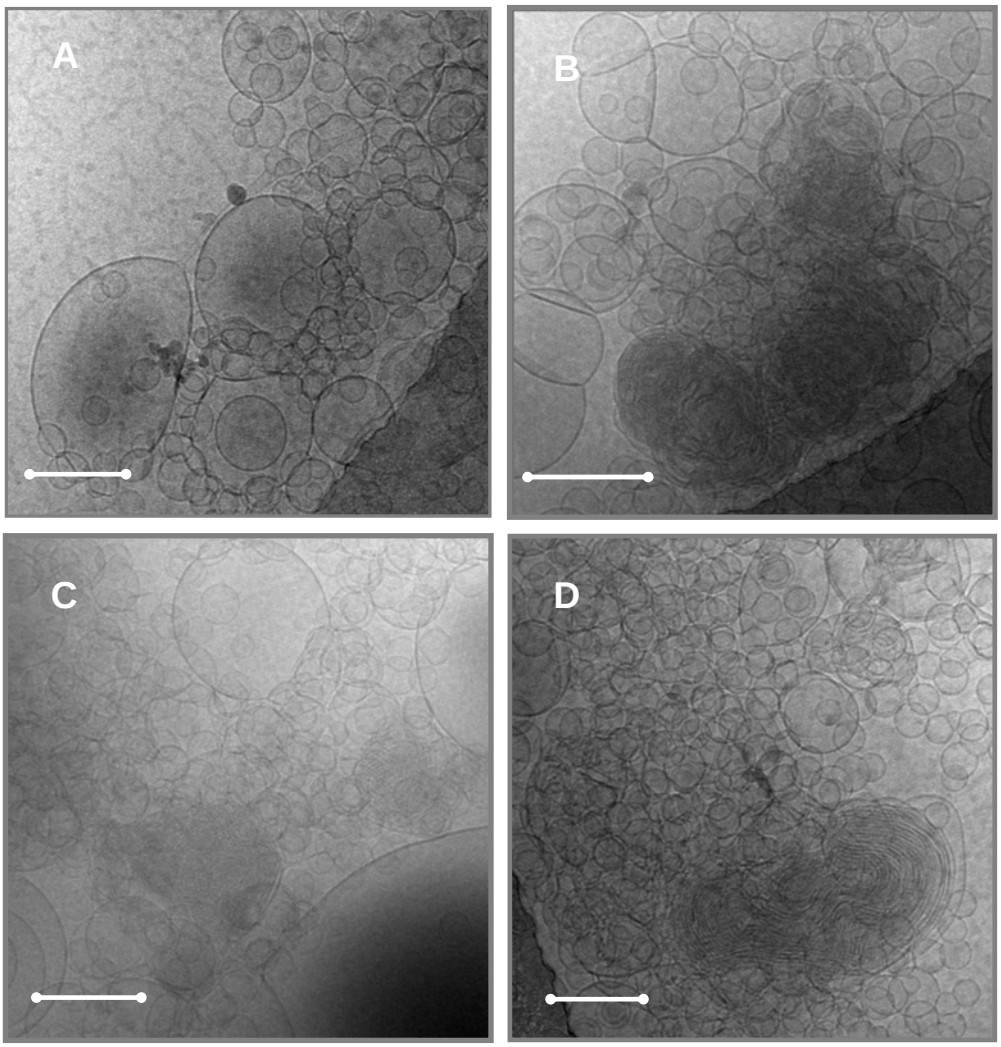

Figure 4.8: Cryo-TEM micrographs of mixed DOTAP/pDNA lipoplexes after interaction with oleic acid (OA)/DOPE/DOPC (1:1:2) liposomes. Negatively charged liposomes were visualized by cryo-TEM immediately after addition to mixed lipoplexes. The mixed DOTAP lipoplexes/OA liposomes were investigated by cryo-EM as described in the Materials and Methods section *2.2.12*. Bars are 200 nm.

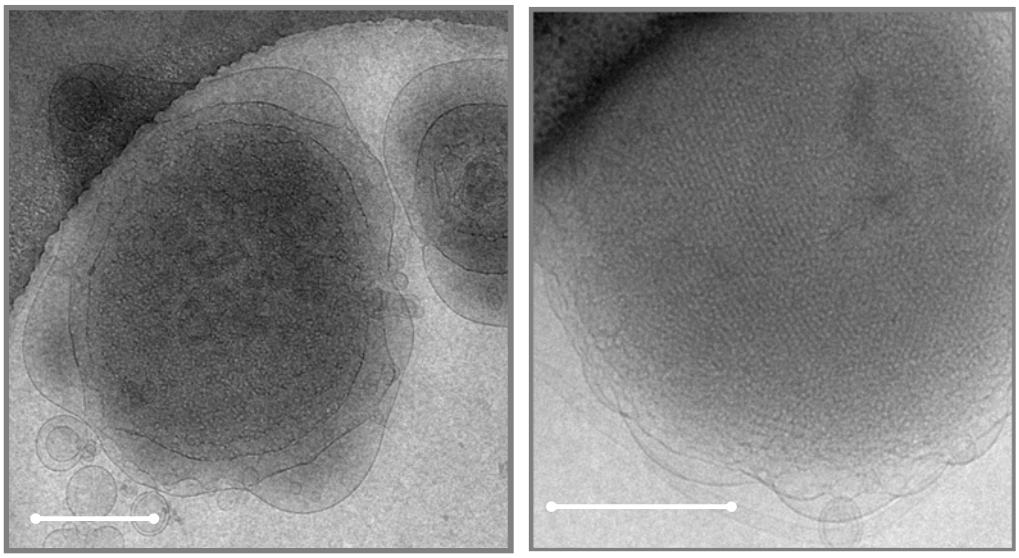

Figure 4.9: Cryo-TEM micrographs of mixed DOTAP/pDNA lipoplexes after interaction with oleic acid (OA)/DOPE/DOPC (1:1:2) liposomes. Anionic liposomes were visualized by cryo-TEM one hour after addition to mixed DOTAP/pDNA lipoplexes. The mixed DOTAP lipoplexes/OA liposomes were investigated by cryo-EM as described in the Materials and Methods section *2.2.12*. Bars are 500 nm.

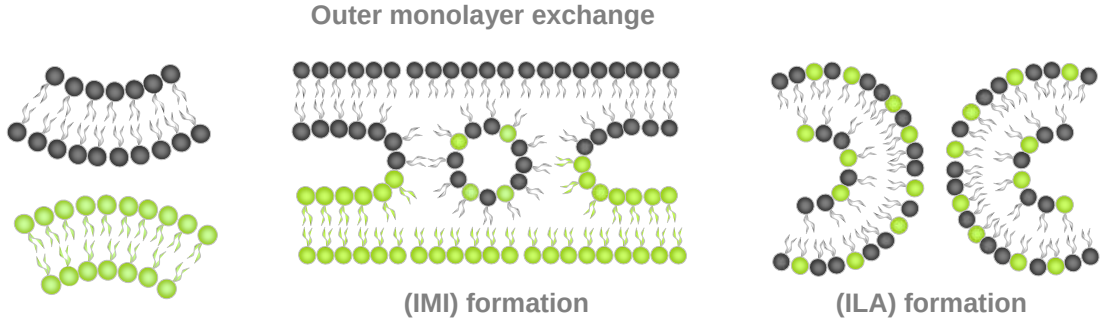

Figure 4.10: Membrane-membrane interactions that can occur via inverted micellar intermediate (IMI) in lipid vesicle dispersions. When IMI form between two vesicles, the outer monolayers of the membranes become continuous, allowing lipid molecules to diffuse back and forth between the two vesicles and formation of Intra-lamellar attachment (ILA) as proposed by (Siegel 1986a).

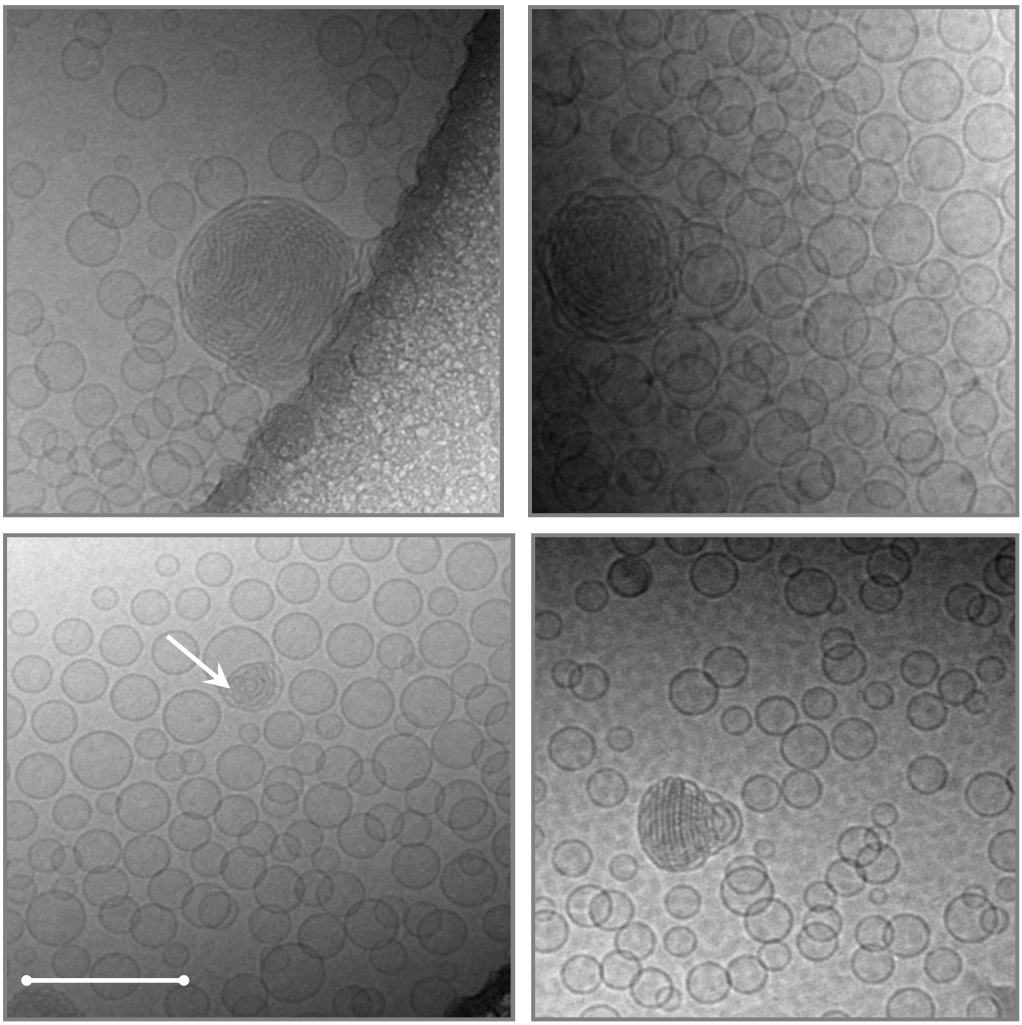

Figure 4.11: Cryo-TEM micrographs of dialysed DOTAP/pDNA lipoplexes after interaction oleic acid (OA)/DOPE/DOPC (1:1:2) liposomes. Anionic liposomes were visualized by cryo-TEM immediately after addition to dialysed DOTAP/pDNA lipoplexes. The dialysed DOTAP lipoplexes/OA liposomes were investigated by cryo-EM as described in the Materials and Methods section *2.2.12*. Bar is 200 nm.

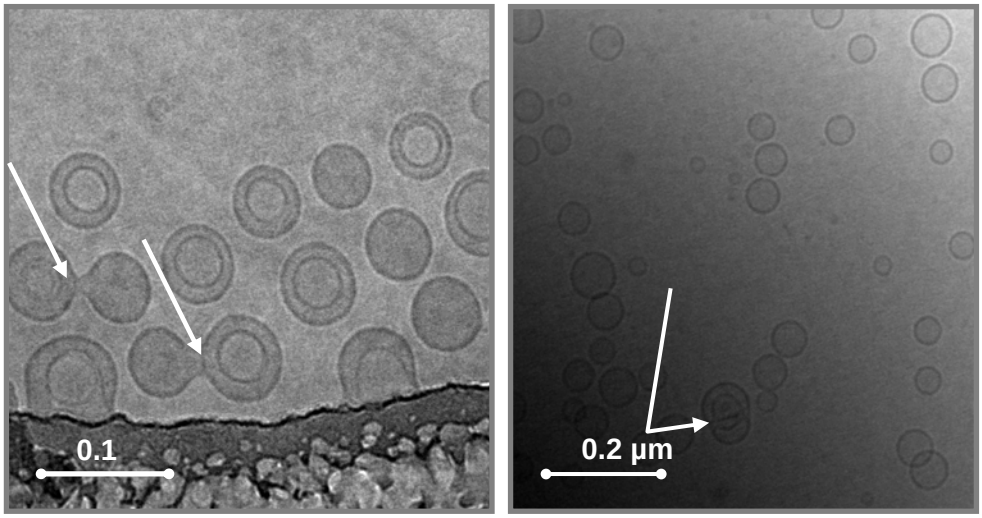

Figure 4.12: Cryo-TEM micrographs of dialysed DOTAP/pDNA lipoplexes after interaction oleic acid (OA)/DOPE/DOPC (1:1:2) liposomes. Anionic liposomes were visualized by cryo-TEM one hour after addition to dialysed DOTAP/pDNA lipoplexes. The dialysed DOTAP lipoplexes/OA liposomes were investigated by cryo-EM as described in the Materials and Methods section *2.2.12*. Bars are indicated.

It is observed that fusion between cationic DOTAP/pDNA lipoplexes and oleic acid liposomes is stimulated by the preference for "inverted" non-bilayer lipid phase structures. We assume that formation of stable "inverted" non-bilayer phase depends on the surface charge neutrality. That means excess free cationic lipids are necessary. It should be noted that at a molar charge ratio of N/P 5, DOTAP/pDNA lipoplexes showed an optimal pDNA wrapping efficiency as reported elsewhere (Madeira et al., 2008). Evidently, at this charge ratio the lipids are in excess over plasmid DNA and, consequently, the phase behaviour described with oleic acid might partly reflect a contribution of free lipids that are not bound to pDNA in mixed lipoplexes. In contrast to dialysed DOTAP/pDNA lipoplexes, these fusion intermediates were rarely found.

However, smaller size of the "inverted" non-bilayer fusion intermediates in comparison to that formed in mixed DOTAP/pDNA lipoplexes was shown (Fig. 4.11).

Some evidences have been reported that the anionic lipids that are more efficient in releasing DNA adopt non lamellar phases with high interfacial curvature when mixed with lipoplexes, while the anionic lipids that are poorly efficient DNA releasers form mostly lamellar phases (Tarahovsky et al., 2004; Koynova et al., 2006; Wang et al., 2006). As evident, our combined nucleic acid dye fluorescence technique and Cryo-TEM results oppose with that opinion.

4.3. Conclusion

In conclusion, the detergent dialysis technique utilized to prepare lipid-based/pDNA complexes showed morphological features which was not observed in case of same mixed lipoplexes. The cationic DOTAP strong compaction effect on pDNA was indicated by the cryo-TEM study.

Cryo-TEM experiments showed that pDNA release from DOTAP/pDNA lipoplexes was mediated by lamellar fusion intermediates formation when DOPS was used, while was mediated by a lamellar/non-lamellar phase transition in mixtures with oleic acid. However, nucleic acid dye fluorescence technique showed that such different phase behaviour did not result in diverse pDNA release efficiency; only the rate of release might be enhanced.

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CHAPTER 5

Membrane Fusion Studies of Lipoplexes Prepared by Detergent Dialysis Technique

Abstract

Our previous study showed that the non-ionic detergent N-octyl-beta-D-glucopyranosid (OGP) dialysis technique utilized to prepare lipoplexes showed neglected pDNA release from DOTAP/pDNA and DC-cholesterol/pDNA lipoplexes when treated with different negatively charged liposomes. In contrast, the extent of pDNA release from these lipoplexes prepared by mixing technique was found to be high. This was accompanied by morphological features, in case of dialysed DOTAP/pDNA lipoplexes, different than that observed in mixed DOTAP/pDNA lipoplexes as visualized by cyro-TEM. These reports were previously described in Chapter 3 and Chapter 4.

Therefore, we estimated in this study if the pDNA release from these lipoplexes is correlated with membrane fusion between lipoplexes and the negatively charged liposomes.

For efficient transfection plasmid DNA has to be released from the cationic lipids and to be translocated into the nucleus. Several investigators have demonstrated that anionic membranes promote cationic lipid/pDNA complex dissociation. They postulated that after endocytosis of the cationic lipid/DNA complex, anionic lipids can migrate from the endosomal membrane into the complex where they displace pDNA from the cationic lipid and promote its release into cytoplasm (Xu and Szoka, 1996; Zelphati and Szoka, 1996; Bhattacharya and Mandal, 1998).

From the literature, it was hypothesised that transfection agents, which are characterized by high transfection efficiency, fused more rapidly with endosomal membranes and escape into the cytoplasm. One finding supports this explanation that increased transfection efficiencies of cationic lipid mixtures correlated well with increased fusion with negatively charged liposomes (Wang and MacDonald, 2004; Koynova and MacDonald, 2007).

Other reports suggested that membrane mixing does not necessarily correlate with the extent of pDNA release from lipoplexes (Zabner et al., 1995; Friend et al., 1996; Wang et al., 2006).

To address this issue that high pDNA release is accompanied by membrane fusion between lipoplex lipids and negatively charged lipid, the membrane destabilization of lipoplexes as a consequence of interaction with negatively charged liposomes was assessed in terms of lipid mixing.

To estimate membrane fusion, (i) octadecyl-rhodamine OD (R18) de-quenching assay (Hoekstra et al., 1984) and (ii) fluorescence resonance energy transfer (FRET) assay (Struck et al., 1981; MacDonald et al., 1999) were applied. Two different negatively charged liposomes were used; (i) DOPS/DOPE/DOPC (1:1:2) liposomes and (ii) oleic acid (OA)/DOPE/DOPC (1:1:2). The increase in the fluorescence of the self quenched fluorophore OD (R18) or of the donor NBD-PE fluorophore thus observed is taken as a measure of fusion.

I. Octadecyl-rhodamine OD (R18) De-Quenching Assay

OD (R 18) labelled lipoplexes with 5 mol % with respect to the total lipid content were prepared by both mixing and non-ionic detergent N-octyl-beta-D-glucopyranosid (OGP) dialysis techniques. At this sufficiently high concentration (5 mol %), a self-quenching of OD (R 18) fluorescence occurs.

Once inserted into the lipid membrane, the fluorescent dye does not dissociate from the membranes by either spontaneous transfer of the free monomer into the aqueous phase or by collional transfer (Hoekstra et al., 1984). Fusion of labelled lipoplexes with anionic liposomes membranes will results in dilution of the probe and concomitant relief of the self-quenching as depicted in Figure 5.1 A.

II. Fluorescence Resonance Energy Transfer (FRET) Assay

In FRET, the emission band of the energy donor fluorophore (NBD-PE) overlaps with the excitation band of the energy acceptor fluorophore (Rh-PE). The excited energy state of the donor generated by an absorbed photon is transferred non-radiatively to the acceptor, which in turn starts to fluoresce. The overlap of the emission spectrum of the donor and the absorption spectrum of the acceptor, as well as the inverse sixth power of the distance between the two fluorophores, determine the rate and efficiency of energy transfer. The assay depends on the dilution of a donor-acceptor pair from ‘‘labelled’’ liposomes to ‘‘unlabelled’’ liposomes as a result of lipid mixing during membrane fusion (Fig. 5.1 B). The efficiency of energy transfer is then decreased.

Briefly, two fluorescent lipids 0.5 mol % each were incorporated into the cationic lipoplexes, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phosphatidylethanolamine (NBD-PE) and rhodamine phosphatidyl-ethanolamine (Rhd-PE). At optimal energy transfer, the emission of the donor (NBD-PE) at λ_{em} 535 nm was strongly suppressed when the excitation wavelength was λ_{ex} 470 nm at 25 °C, which corresponds to NBD-PE absorption (Struck et al., 1981). Fusion of labelled lipoplexes with unlabelled negatively charged liposomes was indicated by an increase in NBD-PE fluorescence because probe dilution and fluorophore separation, and hence, reduced energy transfer efficiency is observed.

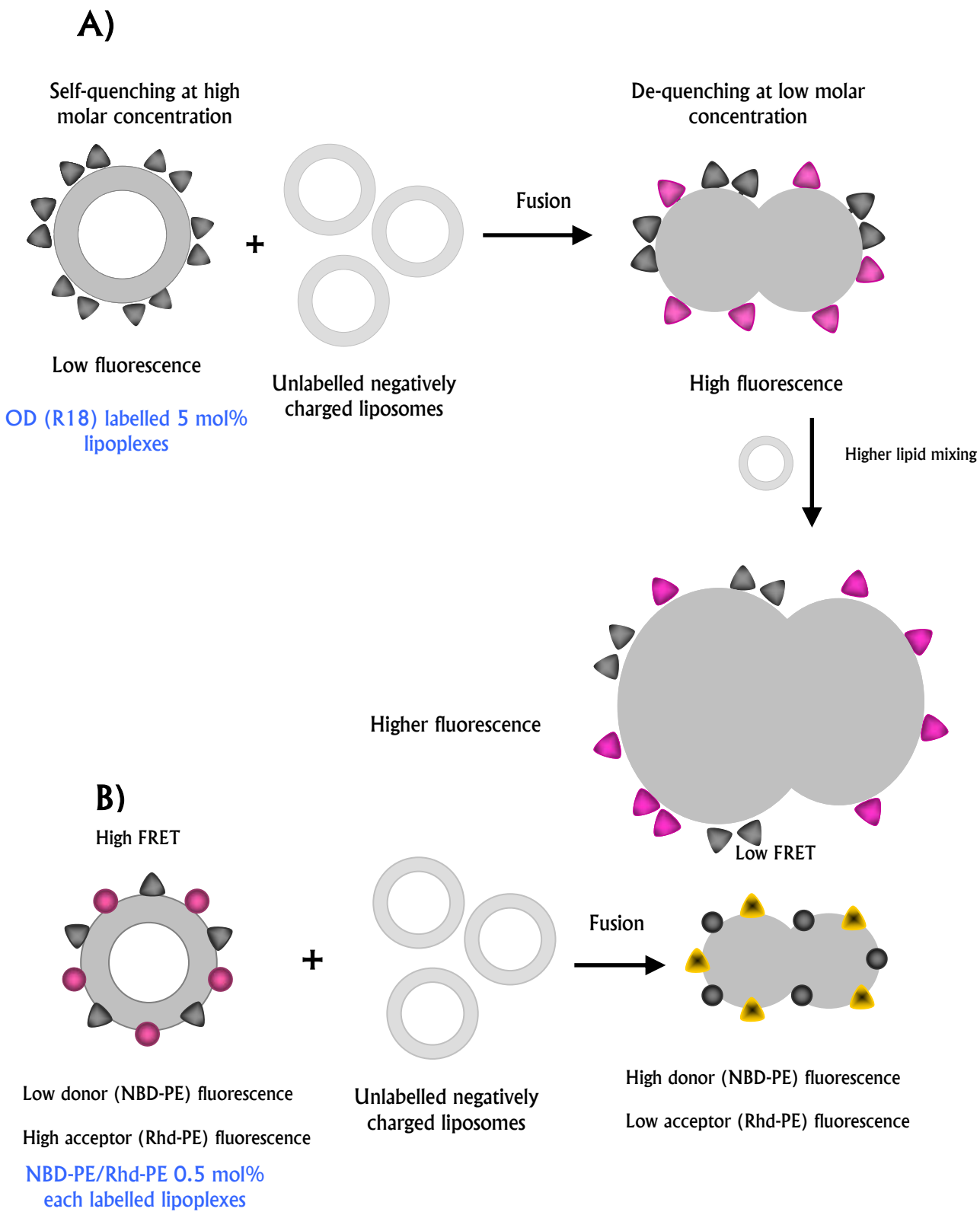

Figure 5.1: Lipid mixing techniques used to estimate membrane fusion. Octadecyl rhodamine (R18) (A) and FRET (B) assays are demonstrated.

5.1. Vesicle-Vesicle Interaction: I. Octadecyl rhodamine OD (R18) De-quenching Dilution Membrane Mixing Assay

5.1.1. DOTAP/pDNA Lipoplexes

Fig. 5.2 shows the lipid mixing of OD (R18) labelled DOTAP/pDNA lipoplexes after addition of different negatively charged liposomes.

The lipid mixing showed 61.82 ± 2.68 % (dialysed) and 59.59 ± 2.75 % (mixed) at R = 2. At R = 4, the lipid mixing increased to 73 ± 1 % (dialysed) and 71 ± 0 % (mixed). Dialysed DOTAP/pDNA lipoplexes showed OD (R18) de-quenching and hence virtually showed the same extent of the lipid mixing as observed in case of mixed DOTAP/pDNA lipoplexes when DOPS/DOPE/DOPC liposomes were used (Fig.5.2 A).

According to pDNA release experiments, dialysed technique generally produced tightly bound cationic lipid/pDNA lipoplexes, as previously shown, compared to mixed lipoplexes. Unexpectedly, dialysed DOTAP/pDNA lipoplexes exhibited an increase in lipid mixing upon addition of DOPS/DOPE/DOPC liposomes regardless to the extent of pDNA release.

In correspondence to membrane fusion assessed by OD (R18) de-quenching assay, the pDNA release from dialysed DOTAP/pDNA lipoplexes by negatively charged lipid DOPS was not correlated with pDNA release from the same mixed lipoplexes.

In cryo-TEM studies we observed that dialysed DOTAP/pDNA lipoplexes and DOPS liposomes showed vesicle aggregation. We assume that, the aggregation might be accompanied by OD (R18) fluorophore dilution. However, the probe dilution between the outer monolayers could also be possible indicating that the probe exchange is sensitive to aggregation.

Conclusively, OD (R18) assay did not indicate full fusion between DOPS/DOPE/DOPC liposomes and dialysed DOTAP/pDNA lipoplexes the condition which is necessary for lipoplex disassembly.

The lack of correlation between lipid mixing and pDNA release from dialysed DOTAP/pDNA lipoplexes may be attributed to the fact that membrane contact between lipoplexes and anionic membranes is not the absolute limiting step in pDNA release. This is also in the line with Zabner et al., (1995), Friend et al., (1996) and Wang et al., (2006) who suggested that membrane mixing does not necessarily correlate with the extent of DNA release from lipoplexes. Our observations may also be accompanied only by the outer monolayers of the aggregated vesicles showing a semifusion process which could also be detected by probe dilution assay.

As shown here, membrane fusion between DOPS/DOPE/DOPC liposomes and dialysed DOTAP/pDNA lipoplexes was as the same extent as mixed DOTAP lipoplexes, although evidently was not related to pDNA released in these systems.

To further confirm the phase dependent destabilization properties of DOTAP/pDNA lipoplexes as detected by cryo-TEM using OA/DOPE/DOPC liposomes, we next investigated the effect of mesomorphic phase formation on DOTAP/pDNA lipoplex disassembly in terms of lipid mixing.

We examined the interaction between the DOTAP lipoplexes and OA/DOPE/DOPC (1:1:2) liposomes. As shown in Fig. 5.2 B, when OA containing liposomes were incubated with DOTAP/pDNA lipoplexes at R = 2 and 4, an extensive lipid mixing for both dialysed and mixed DOTAP/pDNA lipoplexes was also observed. The lipid mixing showed 53.6 ± 0.34 % (dialysed) and 61.77 ± 0.96 % (mixed) at R = 2 as reflected by almost instantaneous increase in OD (R18) fluorescence. R = 4 resulted in similar lipid mixing for both lipoplexes as observed for R = 2. For DOTAP/pDNA lipoplexes treated with DOPS containing liposomes, lipid mixing was anionic/cationic ratio dependent. This was observed, as shown in Figure 5.2 A, by higher lipid mixing when R increased from 2 to 4 than that observed with oleic acid containing liposomes.

5.1.2. DC-cholesterol/pDNA Lipoplexes

The membrane mixing observed in case of mixed DC-cholesterol/pDNA lipoplexes was different from dialysed lipoplexes and correlated well with the pDNA release data. Mixed DC-cholesterol/pDNA lipoplexes showed a marked increase in the probe dilution and a higher de-quenching effect compared to dialysed lipoplexes. Dialysed DC-cholesterol/pDNA lipoplexes showed slower and lesser degree of lipid mixing (Fig. 5.3). At R = 2, DC-cholesterol/pDNA lipoplexes exhibited 12.85 ± 1.23 % (dialysed) and 48.4 ± 1.45 % (mixed) lipid mixing when DOPS containing liposomes were used. A marked increase in lipid mixing was observed when R increased from 2 to 4 showing 19.5 ± 2.95 % (dialysed) and 58.66 ± 2.2 % (mixed) as shown in Figure 5.3 A. These data are consistent with pDNA accessibility measured by PicoGreen® fluorescence assay when the same lipoplexes were treated with DOPS containing liposomes.

The effect of oleic acid, an inverted non bilayer forming lipid, on the DC-cholesterol/pDNA lipoplexes is illustrated in Figure 5.3 B. Lipid mixing was found to be particularly prominent in case of mixed DC-cholesterol/pDNA lipoplex rather than for the dialysed lipoplexes.

The observed extent of lipid mixing was 9.1 ± 0.85 % (dialysed) and 46.8 ±1.64 % (mixed) at R= 2, and 12.1± 3.9 % (dialysed) and 41.15 ± 1.71 % (mixed) at R=4. In contrast to pDNA release data, the extent of lipid mixing after addition of oleic acid liposomes to DC-cholesterol/pDNA lipoplexes was found to be comparable to DOPS/DOPE/DOPC (1:1:2) liposomes.

In conclusion, although the aggregation was accompanied by probe dilution, the chosen conditions were in a range where the DOPS containing liposomes were still not able to destroy the dialysed lipoplexes and induce pDNA release as a consequence.

Taken together, these findings led to the hypothesis that there may be an influence of the detergent substitution technique on the produced lipoplexes. This effect is presumably due to the tight compaction of pDNA in lipoplexes. This could well correlated with low *in-vitro* transfection efficiency reported else where (Guo et al., 2000; Xu et al., 2001; Heyes et al., 2007).

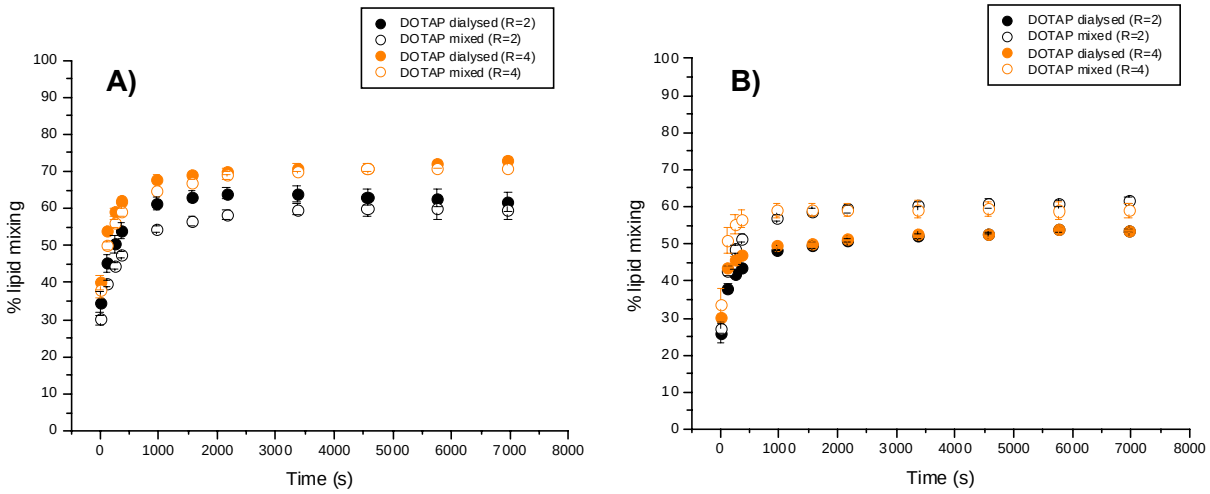

Figure 5.2: Time-dependent effect on lipid mixing between OD (R18) labelled DOTAP/pDNA lipoplexes and negatively charged DOPS/DOPE/DOPC (1:1:2) (A); OA/DOPE/DOPC (1:1:2) (B) liposomes. DOTAP/pDNA lipoplexes prepared at N/P 5 were treated with anionic lipid at R=2 (dialysed (●); mixed (○)) and R=4 (mixed (●) and mixed (○)). Lipid mixing was deduced by OD (R18) de-quenching. Fluorescence intensity was monitored as a function of time with an excitation wavelength λ_{ex} 544 nm and an emission wavelength λ_{em}590 nm, at pH 7.4. The percentage of membrane fusion was calculated according to $\Delta F_t / F_{OGP} = (F_t - F_0)/F_{OGP}$ as described in the Materials and Methods section *2.2.11.1*. Data were shown as mean +/- standard deviation of three independent experiments.

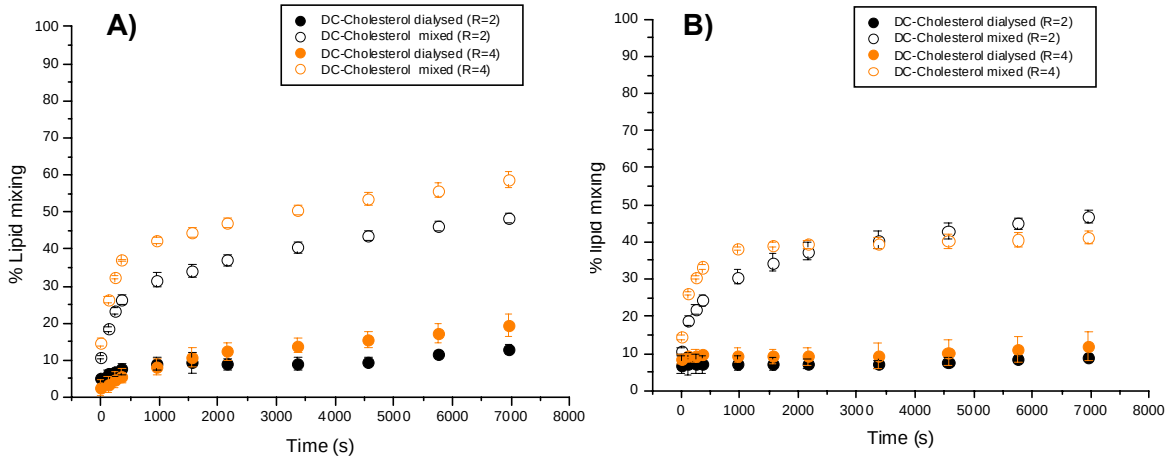

Figure 5.3: Time-dependent effect on lipid mixing between OD (R18) labelled DC-cholesterol/pDNA lipoplexes, and negatively DOPS/DOPE/DOPC (1:1:2) (A) as well as OA/DOPE/DOPC (1:1:2) (B) liposomes. DC-cholesterol/pDNA lipoplexes prepared at N/P 5 treated with anionic lipid at R=2 (dialysed (●); mixed (○)) and R=4 (mixed (●) and mixed (○)). Lipid mixing was deduced by OD (R18) de-quenching. Fluorescence intensity was monitored as a function of time with an excitation wavelength λ_{ex} 544 nm and an emission wavelength λ_{em}590 nm, at pH 7.4. The percentage of membrane fusion was calculated according to $\Delta F_t / F_{OGP} = (F_t - F_0)/F_{OGP}$ as described in the Materials and Methods section *2.2.11.1.* Data were shown as mean +/- standard deviation of three independent experiments.

5.2. Vesicle-Vesicle Interaction: II. FRET Membrane Lipid Mixing Assay

5.2.1. DOTAP/pDNA Lipoplexes

Although the extent of pDNA release from dialysed lipoplexes was low, our previous lipid mixing studies using OD (R 18) showed a marked lipid mixing extent in dialysed DOTAP/pDNA lipoplexes equal to mixed DOTAP/pDNA lipoplexes when negatively charged DOPS containing liposomes were used.

To investigate the lipid mixing characteristics of DOTAP/pDNA lipoplexes in terms of energy transfer efficiency, we performed probe dilution FRET assay to assess the membrane fusion of labelled lipoplexes when different negatively charged liposome were applied.

Fusion between NBD-PE/Rhd-PE- labelled lipoplexes with unlabelled negatively charged liposomes gave rise to a decrease in the surface density of the probes and consequently a relief of donor quenching occurred. In Figure 5.4 and Figure 5.5, a series of experiments with the FRET pair NBD-PE and Rhd-PE labelled lipoplexes at N/P ratio 5 is shown. For each lipoplex, the FRET efficiency decrease as the acceptor concentration decreases, however with various extents.

The experimental results illustrate the overall decrease in FRET efficiency at R = 2. Figure 5.4 A shows extensive FRET efficiency reduction, $E = 1 - I_{DA}/I_D$, for NBD-PE/Rhd-PE pairs among DOTAP/pDNA lipoplexes when treated with DOPS/DOPE/DOPC (1:1:2) liposomes. The initial values of energy transfer efficiency ($E_\circ$) were 21 ± 0.01 % and 18.5 ± 0.01 % for dialysed and mixed

DOTAP/pDNA lipoplexes, respectively, and therefore in a comparable range. This low ($E_\circ$) values in DOTAP/pDNA lipoplexes suggest an instant interaction between DOTAP/pDNA lipoplexes and DOPS/DOPE/DOPC (1:1:2) liposomes as observed in OD (R18) lipid mixing assay. This is in the line with cyro-TEM observations where vesicles aggregations were involved.

The release of pDNA and lipid structural reorganisation shown in DOTAP/pDNA lipoplexes, treated with OA/DOPE/DOPC (1:1:2) liposomes, are interpreted as energy transfer efficiency (Fig. 5.4 B). The initial values of energy transfer efficiencies ($E_\circ$) for oleic acid containing liposomes were comparable for the mixed and dialysed DOTAP/pDNA lipoplexes. An initial energy transfer efficiency of approx. 29% for both dialysed and mixed DOTAP lipoplexes could be observed and the extent of energy transfer efficiencies become distinguished over time showing 11.68 ± 0.01 % for mixed and complete loss of energy transfer efficiency for dialysed lipoplexes (Figure 5.4 B).

In contrast to DOPS, the oleic acid effect on the DOTAP/pDNA lipoplexes was distinguished showed different values by means of initial and extent of energy transfer efficiency (Fig 5.4 A and B).

5.2.2. DC-cholesterol/pDNA Lipoplexes

The energy transfer efficiency data of DC-cholesterol/pDNA lipoplexes revealed a correlation between the extent of membrane fusion of lipoplexes and pDNA release that was induced by negatively charged liposomes.

As shown in Figure 5.5 A, the initial stability of both DC-cholesterol/pDNA lipoplexes in presence of DOPS/DOPE/DOPC liposomes was high compared to DOTAP/pDNA lipoplexes 89 ± 0.01 % (dialysed) and 84 ± 0.01 % (mixed) energy transfer efficiency. After incubation with DOPS/DOPE/DOPC liposomes, a decrease in energy transfer efficiency was observed over time showing 70.4 ± 0.01 % for dialysed lipoplexes whereas mixed lipoplexes showed only 50 ± 0.01 % energy transfer efficacy (Fig 5.5 A).

Although the pDNA release from DC-cholesterol/pDNA lipoplexes by oleic acid was more efficient than that observed using DOPS/DOPE/DOPC liposomes, the initial values of energy transfer efficiency ($E_\circ$) of these lipoplexes in presence of OA showed higher stability to disassemble. From the figure 5.5 B, energy transfer efficiencies of 76 ± 0.02 % (dialysed) and 60.5 ± 0.01 % (mixed) were obtained after incubation with OA/DOPE/DOPC (1:1:2) liposomes. The lipid reorganization during lipoplex dissociation might affect the redistribution of the fluorescent probes keeping the energy transfer efficiency maintained although their ability to exert pDNA release is still efficient.

Lipoplex stability in terms of lipid mixing and disassembly, due to the effect of anionic lipids, was observed for DC-cholesterol/pDNA lipoplexes than DOTAP/pDNA lipoplexes. This could be observed in the initial ($E_\circ$) efficiencies between the probes. These values decreased overtime showing a maximum probe dilution only in case of DOTAP/pDNA lipoplexes where no FRET was found to occur (Fig. 5.4).

In contrast, DC-cholesterol/pDNA lipoplexes showed high initial (E_o) indicating higher stability to disassemble by the effect of negatively charged liposomes (Fig. 5.5).

FRET assay was used previously to assess the stability of pegylated antisense phosphodiester oligonucleotides (ONs) lipoplexes in presence of DNase I (Remaut et al., 2005) and also the condensed state of pDNA in lipoplexes in presence of serum (Itaka et al., 2002).

Recent studies showed that DOTAP/DOPC lipoplexes are very unstable against disintegration by cellular lipids and rapidly release DNA (Caracciolo et al., 2007a; Caracciolo et al., 2007b; Caracciolo and Caminiti, 2009). This binary DOTAP/DOPC lipoplexes showed almost no cytoplasmic plasmid DNA released. When DC-cholesterol was added to this binary complex, lipoplexes were mainly distributed throughout the cytoplasm and to some extent at the cell periphery (Caracciolo and Caminiti, 2009). This reflects a higher stability of DC-cholesterol containing lipoplexes.

A similar observation reported by Berezhna et al., (2005) who visualized the interaction between DOTAP lipoplex and giant uni-lamellar vesicles (GUVs) composed of DOPS/DOPE/DOPC using laser scanning imaging. They found aggregations at the surface of the GUV, but no rupture of the vesicles was detected. Importantly, this aggregation might be indicative of lipid translocation to the outer leaflet of the bilayer as supposed by Kahya et al., (1996).

Our observations may also be attributed to the mixing of lipids located only in the outer monolayers of the aggregated DOTAP/DOPS vesicles (semifusion) showing probe dilution in both OD (R18) and FRET assays.

Previous work showed that lipid mixing does not have to impair bilayer integrity. It could be a lipid exchange at points of close contact that the vesicles could then dissociate (Düzgünes et al., 1987). However, it is also possible to interpret the results in terms of structural changes in the bilayer affecting both the aggregation and fusion steps (Wilschut et al., 1985).

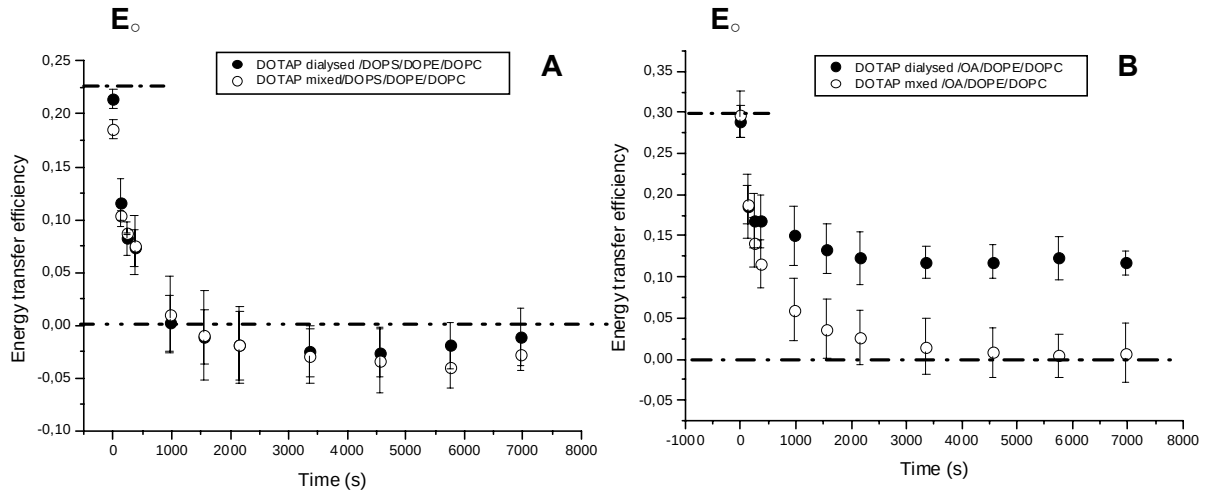

Figure 5.4: Time-dependent effect on energy transfer efficiency (*E*) between NBD-PE/Rhd-PE labelled DOTAP/pDNA lipoplexes, and negatively DOPS/DOPE/DOPC (1:1:2) (A) as well as OA/DOPE/DOPC (1:1:2) (B) liposomes. DOTAP/pDNA lipoplexes prepared at N/P 5 were treated with anionic lipid at R=2 (dialysed (●); mixed (○)). FRET was detected by NBD-PE de-quenching. Fluorescence intensity was monitored as a function of time with an excitation wavelength λ_{ex} 470 nm and an emission wavelength λ_{em}535 nm at 25°C. The energy transfer efficiency was calculated according to E =1- [F_{DA} / F_D] as described in the Materials and Methods section *2.2.11.2.* Data were shown as mean +/- standard deviation of three independent experiments.

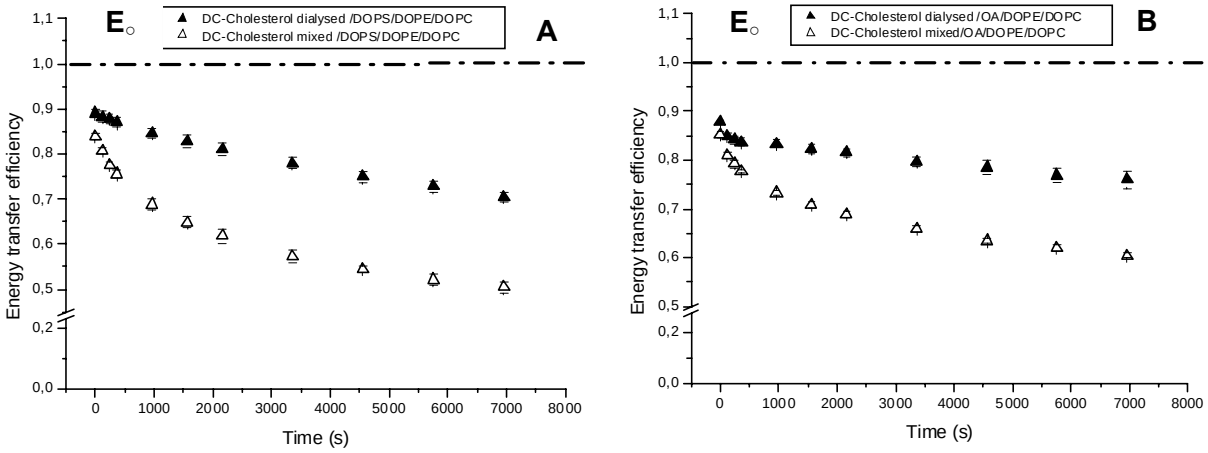

Figure 5.5: Time-dependent effect on energy transfer efficiency (*E*) between NBD-PE/Rhd-PE labelled DC-cholesterol/pDNA lipoplexes, and negatively DOPS/DOPE/DOPC (1:1:2) (A); OA/DOPE/DOPC (1:1:2) (B) liposomes. DC-cholesterol/pDNA lipoplexes prepared at N/P 5 were treated with anionic lipid at R=2 (dialysed (▲); mixed (Δ)). FRET was deduced by NBD-PE de-quenching. Fluorescence intensity was monitored as a function of time with an excitation wavelength λ_{ex} 470 nm and an emission wavelength λ_{em}535 nm at 25°C. The Energy transfer efficiency was calculated according to E =1- [F_{DA} / F_D] as described in the Materials and Methods section *2.2.11.2.* Data were shown as mean +/- standard deviation of three independent experiments.

5.3 Conclusion

In conclusion, octadecyl rhodamine B self-quenching as well as FRET were used to study the dissociation of lipoplexes by negatively charged liposomes in terms of membrane fusion with lipoplexes. It turned out that membrane fusion study using octadecyl rhodamine B de-quenching assay gave data which were comparable to data from the FRET assay.

General observations in our experiments with DOTAP/pDNA lipoplexes were the following: (i) no correlation observed between dialysed lipoplexes pDNA release and lipid mixing with different negatively charged liposomes. This was indicated by virtual membrane fusion compared to mixed DOTAP/pDNA lipoplexes using both OD (R18) and FRET; (ii) the apparent high lipid mixing could be presumably due to probe dilution among the outer monolayers of the aggregated vesicles as indicated by the cryo-TEM studies and (iii) DOTAP/pDNA lipoplexes showed considerably low energy transfer efficiencies immediately upon mixing with anionic lipids the condition indicates lower lipoplexes stability and higher disassembly.

DC-cholesterol/pDNA lipoplexes showed a well correlation between the membrane fusion with negatively charged liposomes and pDNA released as observed by both techniques.

The differences between the PicoGreen® and the fusion assays may be related to the fact that the probe dilution fluorescence assays do not directly relate to pDNA release behaviour as shown in the pDNA release section.

5.4. References

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CHAPTER 6

Characterization of Polyethylenimine/pDNA Polyplexes Prepared by Detergent Dialysis Technique

Abstract

Complexes of plasmid DNA (pDNA) and the 25 kDa branched polycationic polymer polyethylenimine (PEI) are usually prepared by simple mixing of both components, leading to complex formation based on electrostatic interactions. In the present study, polyplexes were formed by an alternative technique complexing pDNA and PEI in the presence of the non-ionic detergent N-octyl-beta-D-glucopyranosid (OGP) at different N/P ratios followed by dialysis. The mixed and dialysed polyplexes were characterized by laser light scattering to determine the influence of OGP on the hydrodynamic diameter and the zeta potential of the particles. Furthermore, using the PicoGreen® assay the status of pDNA condensation was determined. Gene expression of the polyplexes was characterized in HEK 293 cells using flow cytometry analysis.

The preparation technique influenced the hydrodynamic diameter, zeta potential as well as de-condensation of pDNA from the polyplexes dependent on the OGP concentration used. The preparation technique influenced physicochemical as well as biological results. With increasing OGP concentration zeta potential of the dialysed complexes decreased. This effect was correlated with an increase in polyplex size suggesting a tendency of the dialysed particles to aggregate. Dialysed polyplexes were characterized by a stronger condensation of pDNA compared to mixed PEI polyplexes. Corresponding to the physicochemical characteristics, the dialysed polyplexes showed lower expression of green fluorescent protein in HEK 293 cells compared to the mixed polyplexes.

6.1. Size and Zeta Potential of Polyplexes

Hydrodynamic diameter and zeta potential of mixed and dialysed polyplexes were determined by photon correlation spectroscopy and laser Doppler anemometry, respectively. For these measurements, mixed polyplexes of 25 kDa branched polyethylenimine were prepared with plasmid DNA at different N/P ratios in 10 mM Tris buffer solution at pH 7.4 by simple mixing of both components leading to complex formation based on electrostatic interactions. Additionally, dialyzed polyplexes were formed by an alternative technique complexing pDNA and PEI in the presence of the non-ionic detergent OGP followed by dialysis. OGP concentrations were varied from 50 to 200 mM as detailed in the figures. All chosen concentrations were higher than the critical micellar concentration (CMC) of OGP. All polyplexes were freshly prepared before experiments at two different N/P ratios 5 and 10. These ratios were chosen based on previously published experiments with mixed polyplexes made of 25 kDa branched PEI (Fischer et al., 1999) leading to condensed, stable and highly effective polyplexes. For details of preparation see chapter 2 Materials and Methods section 2.2.3.

Size and zeta potential of polyplexes were found to be dependent on (i) preparation technique of polyplexes, (ii) N/P ratio, and (iii) OGP concentration used for polyplex preparation.

More specifically, the PEI/pDNA polyplexes formed by the mixing technique (0 mM OGP) showed comparable hydrodynamic diameters of 111 ± 7.3 nm and 161.7 ±12.7 nm at N/P 5 and 10, respectively (Figure 6.1). This is in accordance to the literature where sizes in the range of <200 nm were reported by several authors for PEI polyplexes produced under the same conditions (Kircheis et al., 2001; Kunath et al., 2003).

Formation of polyplexes in the presence of 50 mM OGP resulted in a slight increase in size to about 203.67 ± 3.69 nm and 190.5 ± 22.29 nm at N/P 5 and 10, respectively, compared to mixed polyplexes without any major difference between N/P 5 and 10. Higher OGP concentrations yielded larger complexes with a small difference between 100 mM OGP (459 ± 58.57 nm) and 200 mM OGP (398 ± 27.01 nm) at N/P 5. At N/P 10 particles generated in 100 mM OGP showed sizes comparable to lower OGP concentrations (180.4 ± 26.12 nm), whereas at 200 mM OGP a significant increase up to 670 nm was observed.

Additionally, the polydispersity indices (PDI) of the size measurements (Figure 6.1) were determined as a marker for particle size distribution. Mixed polyplexes showed a monomodal peak profile with narrow size distribution at N/P 5 and 10 demonstrating a homogeneous size distribution. At the same nitrogen/phosphate ratios dialysed polyplexes were characterized by a wider size distribution often with a bimodal peak profile suggesting aggregation of the particles. The polydispersity index increased with increasing OGP concentration.

Interactions between PEI/pDNA polyplexes and negatively charged cell surfaces are based on electrostatic interactions and are therefore dependent on the particle surface charge (Boussif et al., 1995). The results of the zeta potential analysis for PEI/pDNA polyplexes are depicted in Figure 6.2. Mixed polyplexes showed comparably high positive zeta potentials of 31.8 ± 0.64 mV and 34.3 ± 2.48

mV at N/P 5 and 10, respectively. The values of these data correspond well to previous publications (Boussif et al., 1995; Godbey et al., 1999) reporting zeta potentials in a comparable range of about 30 mV.

In contrast, at the same N/P ratios all dialysed polyplexes showed lower zeta potential values than the mixed polyplexes (Figure 6.2). Zeta potentials decreased with increasing concentration of OGP in the range from 50 mM to 200 mM. This effect was more pronounced for polyplexes prepared at N/P 5 than at N/P 10. Lowest zeta potential values were observed for dialysed polyplexes prepared with 100 mM (4.23 ± 0.71 mV) and 200 mM (7.77 ± 0.77 mV) OGP at N/P 5. For comparison, the zeta potential of free pDNA formulated only with OGP and without polymer was determined (Figure 3.8). Naked pDNA exhibited a negative charge of -51.4 ± 1.76 mV. Higher OGP concentrations decreased the zeta potential up to

-20.01 ± 3.31 mV for 200 mM OGP.

Size and zeta potential of pDNA/polycation complexes are important parameters that should control not only hemocomatibility, the deposition and body distribution of polyplexes after their in vivo administration, but also their uptake by target cells. Sizes smaller than 150-200 nm and zeta potentials in the range from 30-40 mV were found to accomplish cellular interaction and uptake through endocytosis (Park et al., 2000). Furthermore, also the velocity of cytoplasmatic movement was found to be a function of particle size (Park et al., 2000). Mixed polyplexes of pDNA and 25 kDa PEI complexed at N/P 5 and 10 showed physicochemical properties in this desired range. The ability of the PEI polymer to sufficiently neutralize the negative charges of pDNA for the formation of an adequately compacted structure increased with higher N/P ratios (Erbacher et al., 1999). The influence of the N/P ratio on complex size has been described by others (Ogris et al., 1998). In our experiments, two different N/P ratios 5 and 10 were used which both were previously shown to produce condensed polyplexes with no significant difference in size.

The detergent dialysis technique was shown to produce pDNA/lipid particles which were highly stable compared to those produced by mixing technique (Reimer et al., 1995; Zhang et al., 1997). Consequently, in the present study this technique was transferred from lipoplexes to polyplexes of pDNA and 25 kDa branched PEI.

Concluding our laser light scattering experiments of dialysed polyplexes, the increase in hydrodynamic diameter correlated well with a decrease in zeta potential suggesting a tendency of the dialysed polyplexes to aggregate. This could be based on the lower cationic particle surface charge at higher OGP concentrations and therefore, a reduced electrostatic repulsion of the particles.

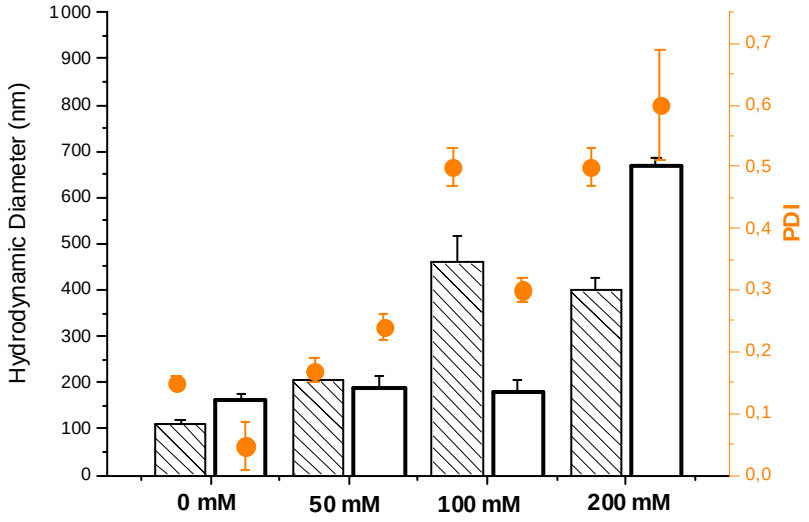

Figure 6.1: Influence of OGP concentration on the hydrodynamic diameter of polyplexes at different N/P ratios. The effect of different OGP concentrations (0-200 mM) on the hydrodynamic diameter and poly-dispersibility index (PDI) of polyplexes at N/P 5 (striated bars) and N/P 10 (solid bars) was measured by photon correlation spectroscopy in three independent experiments. Results are given as mean +/- standard deviation.

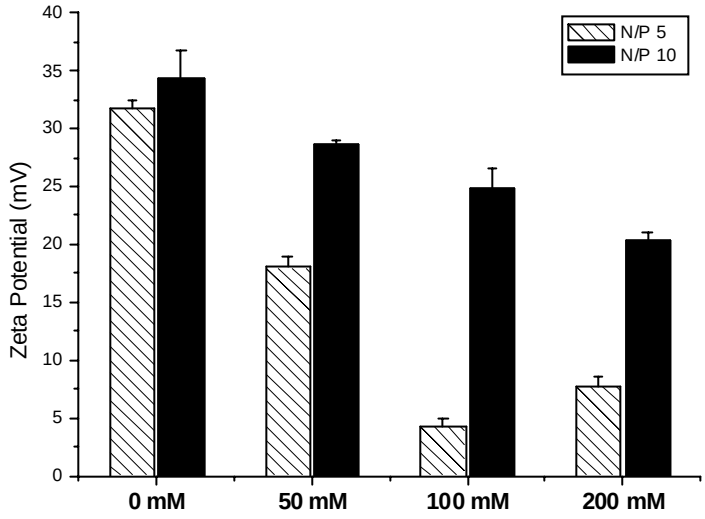

Figure 6.2: Effect of OGP concentration (0-200 mM) on the zeta potential of polyplexes at different N/P ratios. The zeta potential of polyplexes at two different N/P ratios (N/P 5, striated bars and N/P 10, solid bars) was measured by laser Doppler anemometry in three independent experiments. Results are shown as mean +/- standard deviation.

6.2. pDNA Condensation

The condensation of polyanionic pDNA by the cationic charges of PEI into small particles is an important prerequisite for gene delivery (Dunlap et al., 1997). The aim of the pDNA condensation experiments was to quantify the condensed or uncondensed status of pDNA in polyplexes prepared by detergent dialysis technique relative to polyplexes prepared by the mixing technique. Therefore, experiments were performed with 25 kDa branched PEI and plasmid DNA in the presence of the non-ionic detergent OGP of concentrations in the range from 50-200 mM followed by dialysis at N/P ratios 5 or 10. It was described that plasmid condensation by 25 kDa branched PEI was completed at N/P ratios well above 4 (Kunath et al., 2003; Choosakoonkriang et al., 2003; Kleemann et al., 2004).

Experiments were performed at different pH (8 and 11.6). At alkaline pH, pDNA has been recently reported to be released from PEI polyplexes due to a reduced protonation of PEI compared to the physiological pH 7.4 (Moret et al., 2001). To quantify the amount of uncondensed pDNA, the fluorescent nucleic acid dye PicoGreen® was used. The fluorescence of PicoGreen® is enhanced upon intercalation into pDNA and quenched when displaced by higher affinity compounds. The fluorescence emitted upon addition of the dye to polyplexes was measured by fluorescence spectroscopy. Results were provided as relative fluorescence intensity where 1 is attributed to the fluorescence of PicoGreen® with naked pDNA at that pH. The recovered fluorescence was recorded as a function of the N/P ratio and OGP concentration at alkaline pH.

The intercalation of PicoGreen® into polyplexed pDNA was measured at pH 8 and 11 (Figure 6.3).

At pH 8, in all cases, fluorescence was lower compared to the values measured at pH 11.6. For mixed polyplexes at N/P 5, 18.3 ± 0.81 % fluorescence has been recovered, whereas at the same N/P ratio for OGP-based polyplexes the values were in the range from 3.75 ± 0.29 % (200 mM OGP) to 5.1± 0.12 % (50 mM OGP). Additionally, N/P dependency could be observed since condensation of pDNA was more pronounced at N/P 10 than at N/P 5. Higher N/P ratios of 10 resulted in a higher reduction of the dye accessibility to plasmids compared to N/P 5 ratios (Figure 6.3 A).

For mixed polyplexes at N/P 5 and 10 up to 80-85 % PicoGreen® fluorescence could be recovered at pH 11.6 (Fig. 6.3 B). This corresponds to results previously published by Moret et al. (2001).

In contrast, all polyplexes which were formed in the presence of 50-200 mM OGP at N/P 5 and 10 almost completely suppressed the fluorescence to levels lower than 30 %. The lowest recovered fluorescence (3.6 ± 0.5 %) and therefore highest inaccessibility of PicoGreen® to pDNA could be achieved using 200 mM OGP for the formation of polyplexes at N/P 10.

The most prominent feature of PEI is its high cationic charge density. Every third atom of PEI is a possibly protonable nitrogen atom, which leads to a very high cationic charge density. Since PEI does not contain quaternary amines, cationic charges are generated by protonation of the amine groups by the biological environment, thus leading to a correlation between environmental pH and cationic charge density. PEI showed a level of protonation at pH 7.4 of 20 % compared to about 45 % at pH 5 (Suh et al., 1994). The degree of protonation plays a critical role in polyplex formation, since

interactions with pDNA are based on electrostatic interactions. Also toxicity and endosomal release are a function of pH and buffer capacity. Increasing, in our experiments, the pH from physiological values (pH 7.4) where polyplexes were formed, to alkaline pH 8 and 11.6 reduced the protonation of PEI and therefore the electrostatic interaction with the negatively charged pDNA. Consequently, the pDNA was released from the polyplexes with higher pH and became accessible for the intercalating dye PicoGreen®. Since naked pDNA gave comparable results it was suggested that most of the plasmid has been released and de-condensed in the case of mixed polyplexes. In contrast, the low intercalation of PicoGreen® into pDNA in the presence of OGP suggested a highly condensed state of the pDNA in the polyplexes.

The observed effects could be explained by two different theories: Firstly, as proposed previously that the ionic conditions of the surrounding media critically affect the interaction of multivalent cations with pDNA. Wilson and Bloomfield (1979) observed that the critical concentration of multivalent cations inducing pDNA condensation increases with increasing salt concentration.

According to the counter-ion condensation theory (Manning, 1978; Taira et al., 2005), about 70 % of the negative charge of pDNA is neutralized owing to condensation of the counter-ions in solution. The formation of a condensed counter ion layer due to monovalent ions association is depicted in Figure 6.4. It is believed that the degree of counter-ion condensation is not sensitive to changes in the bulk concentrations of monovalent ions. Thus, a considerable amount of monovalent cations are condensed around pDNA.

The strong compaction of PEI/pDNA in the presence of OGP could be explained as follows based on the literature. Detergents are well known destroyer of the water structures (Gregoriadis, 2007).The presence of non-ionic detergent OGP was suggested to interfere with the distribution of the rigid condensed counter ions around the pDNA as well as the hydration shell altering the spatial water structures. This leads to a higher electrostatic attraction of pDNA to the cationic PEI in aqueous solution than for mixed PEI/pDNA polyplexes where the presence condensed ions layer should be overcome.

In absence of OGP, it would be according to counter-ion condensation that the wrapping of PEI polymer around pDNA may not necessarily neutralize all the pDNA charges but may cause local bending the pDNA required to facilitate condensation.

In accordance with a previous study using fluorescence correlation spectroscopy (FCS) with two-photon-excitation showing that polyplexes contained on average about 3.5 plasmid DNA and 30 PEI (25 kDa) molecules, while a high proportion of polymer (~86 %) remained in its free, unbound form (Clamme, 2003).

The free polymer may play a significant part in cytotoxicity. Nevertheless, an excess of polycation is necessary, because the cationic surface charge of polyplexes mediates the interaction with the negatively charged cell membrane (Labat-Moleur et al., 1996)

According to our zeta potential measurements, the effect of OGP could also be observed on the zeta potential values of pDNA. The zeta potential measurements reflects that the decline of negative zeta potential values of pDNA in presence of OGP as consequence of electric layer disturbance (Figure 3.8). In addition, the effect on the zeta potential values was OGP concentration dependent.

Secondly, the results can also be explained by residual OGP in the polyplexes possibly not fully removed during the dialysis step. The OGP concentration in the dialysis medium could not be measured due to the high dilution. Therefore, values were lower than the detection limit. Suggesting that residual OGP was attached to the pDNA, the lower zeta potential of the pDNA/OGP complex as measured in our experiments (Figure 3.8), limit the electrostatic interaction with PEI. Therefore, lower amounts of PEI could be interacted with the pDNA and conclusively, the zeta potential of the dialysed polyplex is reduced. Furthermore, an attachment of OGP not to the pDNA, but to the surface of the final polyplex will generate the same results.

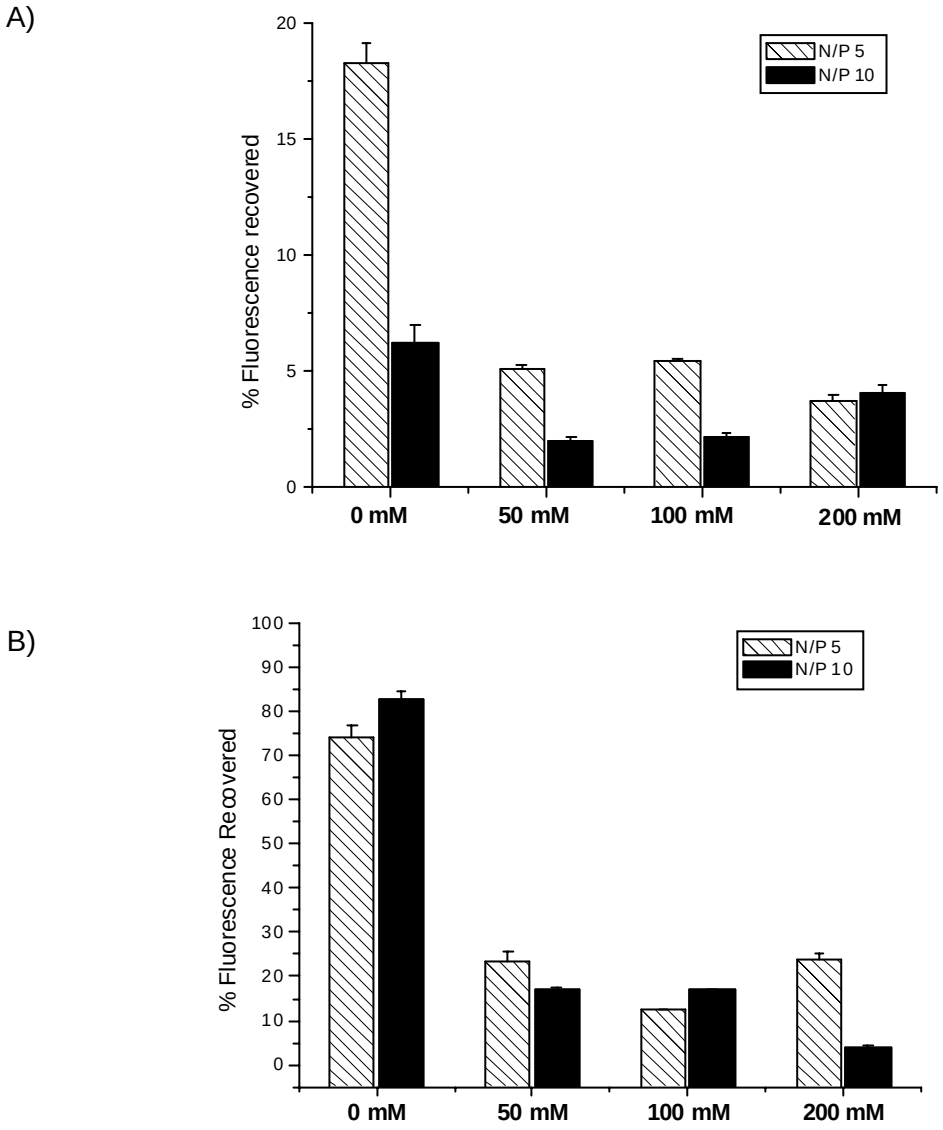

Figure 6.3: Comparison of PicoGreen® fluorescence of polyplexes in dependency of OGP concentration and N/P ratio at pH 8 (A) and 11.6 (B). Polyplexes prepared in OGP containing solutions (0-200 mM) at N/P ratios 5 and 10 were incubated with PicoGreen® for 5 min at pH 8 and 11.6. Fluorescence was measured by fluorescence spectroscopy at λ_{ex} 485 nm and λ_{em} 520 nm. Values are given as mean ± standard deviation (*n*=4).

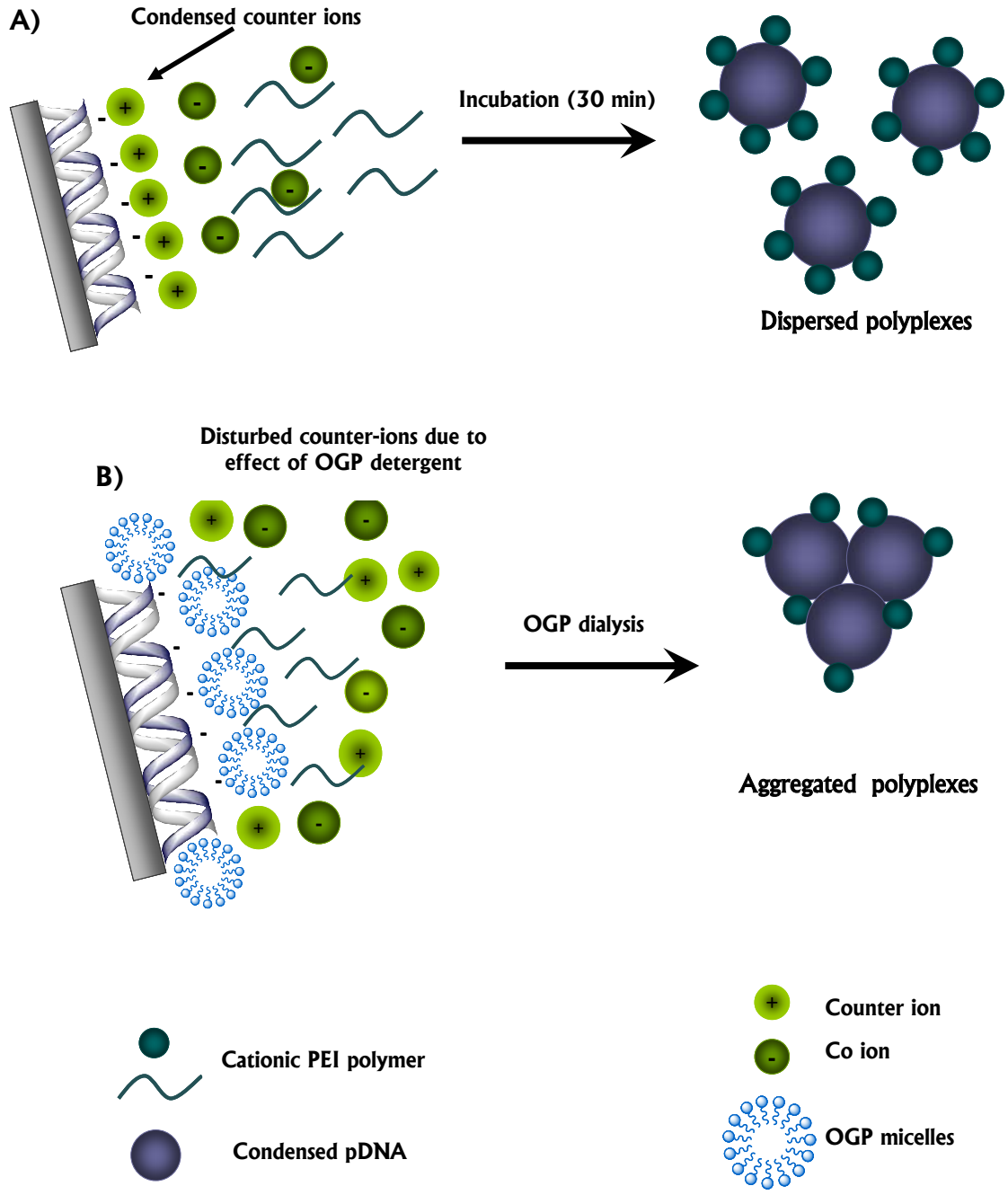

Figure 6.4: Strategies in PEI/pDNA polyplex preparation. The polyplex is prepared by mixing technique (A) producing dispersed particles with highly positive zeta potential values. An alternative approach, illustrating the non-ionic N-octyl-beta-D-glucopyranosid (OGP) detergent dialysis technique. The pDNA is mixed with PEI polymer in the presence of (OGP) followed by dialysis (B). Here the cationic polymer PEI should efficiently condense DNA into polyplexes upon detergent substitution using dialysis technique. This forms aggregated polyplexes characterised by lower zeta potential values and large hydrodynamic diameters.

6.3. Transfection Efficiency

The potential of mixed and dialysed polyplexes to introduce exogenous pDNA into eukaryotic cells was investigated in transfection experiments with regard to polyplex preparation technique, N/P ratio, transfection time and amount of plasmid DNA. HEK 293 cells were cultured in 6-well plates and were transfected *in-vitro* using a plasmid encoding the reporter gene green fluorescent protein (GFP). The transfection efficiency was measured by counting the percentage of GFP positive cells using flow cytometry. Transfection efficiency is given as percentage of cells expressing GFP. Mixed and dialysed polyplexes were applied for 5 h at N/P 5, 10, 15 and 20 using 2 µg and 3 µg pDNA per well. GFP expression was quantified after 24 h and 48 h.

Figure 6.5 A, shows that naked pDNA was not able to transfect the HEK 293 cells effectively. Less than 0.05 % GFP positive cells were detected which was comparable to untreated cells. When HEK 293 cells were treated with mixed polyplexes of different N/P ratios and pDNA amounts (Fig. 6.5 B), transfection levels increased up to 47.1 ± 1.8 % GFP positive cells at 3 µg pDNA and N/P 10. At higher N/P ratios (15 and 20) a significant decrease in transfection efficiency could be observed independent on the amount of pDNA added most likely due to cytotoxic effects of PEI. Phase contrast microscopy revealed changes in the cells morphology. This observation corresponds to the report of Fischer et al., (1999). A high cytotoxicity was observed caused by the high affinity of the polymer to bind the outer surface of the plasma membrane. This was detected using transmission electron microscope by huge clusters of the PEI polymer depending on the size, structure and molecular weight of the polymer. These cluster considered as the main cause of cell necrosis seen after 30 min incubation.

Using dialysed polyplexes the transfection efficiency increased with higher N/P ratios and amount of pDNA as described before for the mixed polyplexes. Treatment of cells with dialysed polyplexes (Fig 6.5 A) demonstrated the highest number of GFP positive cells (3.2 ±0.07 % and 3.9 ± 0.38 %) at N/P 10 and N/P 20 respectively when 3 µg pDNA was applied. This effect needs further investigation. For all dialysed polyplexes the levels of GFP expression were significantly lower than for mixed polyplexes. But all values were higher than for naked pDNA. An increase in transfection time from 24 h to 48 h did not change the transfection rate for N/P 5 polyplexes (Figure 6.6). However, longer transfection times resulted in higher transfection rates for polyplexes at N/P 10.

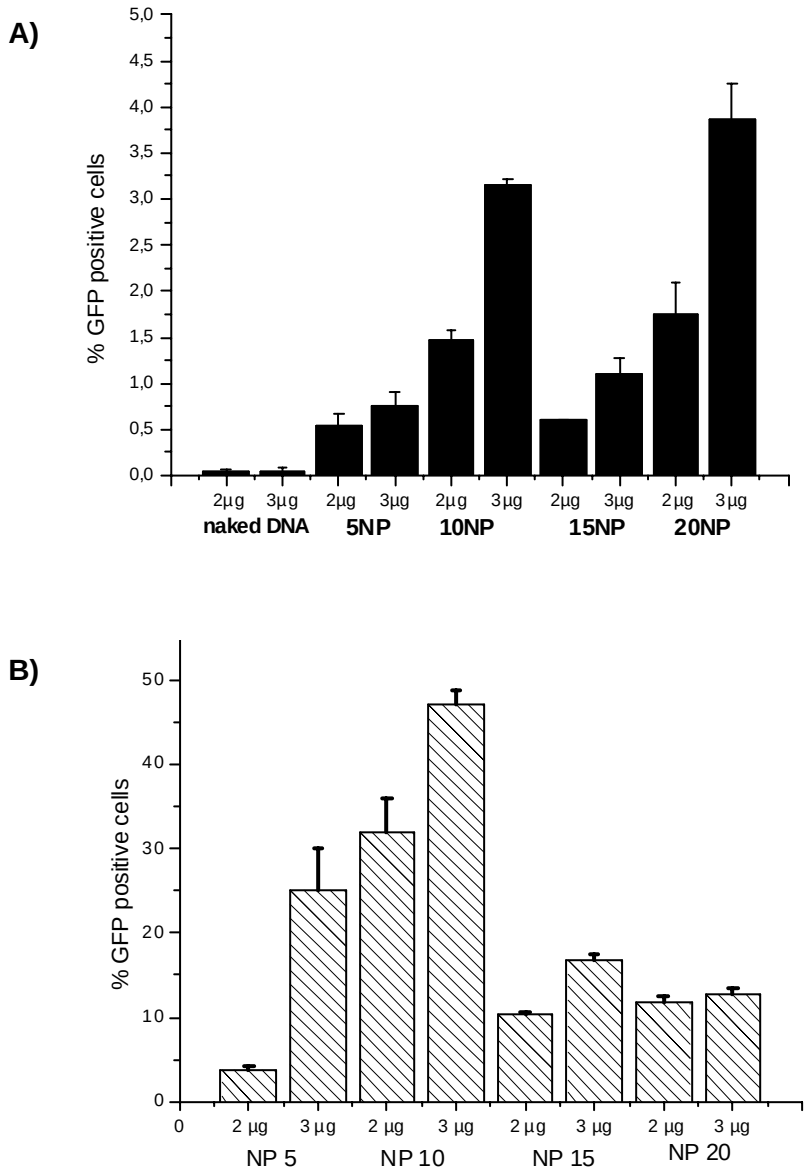

Figure 6.5: Green fluorescence protein (GFP) gene expression of HEK 293 cells after treatment with dialysed (100 mM OGP) (A) and mixed (B) polyplexes at different N/P ratios from 5 to 20. Cells were treated with polyplexes containing 2 and 3 µg/well pDNA. The green fluorescence protein was quantified by flow cytometry 24 h after transfection. Values are shown as mean of six independent experiments. Error bars represent standard deviation.

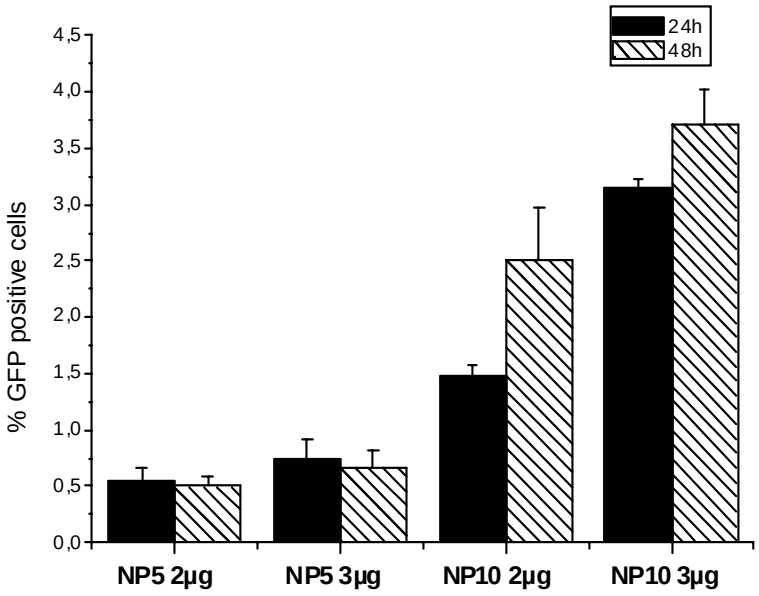

Figure 6.6: Time-dependency of GFP gene expression of dialysed polyplexes. Dialysed polyplexes were prepared in 100 mM (B) OGP at N/P ratios of 5 and 10 followed by dialysis. The green fluorescence protein was assessed by flow cytometry 24 h (solid bars) and 48 h (striated bars) after transfection. The experiment was performed using HEK 293 cells which were treated with 2 and 3 µg pDNA/well. Values were calculated as mean of six independent experiments. Error bars represent standard deviation.

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6.4. Conclusion

In conclusion, transfection data correlated well with the physicochemical data of mixed and dialysed polyplexes. Mixed polyplexes were found to be very successful in transfecting cells due particles sizes smaller than 200 nm and high positive zeta potential which accomplish strong electrostatic interactions with and uptake into eukaryotic cells. For the significantly larger sized dialysed polyplexes with lower zeta potentials, transfection was found to be reduced presumably due to a lower interaction with the negatively charged cell membranes and consequently a lower uptake into the cells. An excess of cationic polymer and a positive surface charge of polyplexes is required to provoke endosomal membrane damage and release of polyplexes (Bieber et al., 2002; Boeckle et al., 2004). Other studies showed that poly (ethylene) glycol derivatives masked the cationic surface charge of PEI/pDNA polyplexes and apparently interferred with the uptake mechanism (Fella et al., 2008). Additionally, the stronger condensation of pDNA and lower release of pDNA from the polyplexes as observed in the condensation experiments for dialysed complexes may also contribute to the low transfection efficiency.

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CHAPTER 7

Conclusive Summary

7. Conclusive Summery

7.1. Conclusive Summery

The potential use of genes as therapeutic agents has attracted attention as a novel approach to the treatment of severe diseases. In the case of inherited disorders, the introduction of a normal copy of the affected gene can be effective, as in the well known case of gene therapy for severe combined immune deficiency due to adenosine deaminase (ADA) deficiency, in which the normal gene for ADA is used to treat the affected patient. For the treatment of acquired disorders, such as cancer and infectious diseases, effective potential strategies involve not only the introduction of a therapeutic gene, such as the gene for a cytokine or an antigen, but also the silencing of the expression of an abnormal gene, whose expression is enhanced in the tissue of the diseased part. Down-regulation of a gene can be achieved by the transfer of short antisense oligonucleotides (ODNs) into cells or by intracellular delivery of the more recently developed small interfering RNA (siRNA). In addition, a gene can be expressed by translocating plasmid DNA into cells containing the sequence for a gene of interest under the influence of a promotor.

Non-viral vectors that comprise cationic lipids and cationic polymers have been found to be safer, easier to prepare and readily chemically modified than viral carriers. The efficiency of these vectors, however, is lower than that of viral vectors and has until now precluded their extensive use for *in-vivo* therapeutic applications. Therefore a better understanding of the parameters that govern transfection efficiency of non-viral vectors as well as the investigation of design and preparation technique of the non-viral carriers will be crucial for their development for *in-vivo* applications.

Self-association between pDNA and complexing agents is accomplished in solution by direct mixing of both components. This approach relies on kinetic control to permit small particles that consist of cationic lipids or polymer and pDNA. In the direct mixing approach, kinetic control is achieved by adjusting the concentration, charge ratio, ionic strength of the solution and rate of mixing of both components. In more sophisticated method, the components are kept from aggregation using the non-ionic detergent N-octyl-beta-D-glucopyranosid (OGP) to disrupt the bilayer structure and to form mixed micelles. This reduces or eliminates the multivalent interaction between the cationic lipid and the pDNA. The detergent is removed by dialysis and as the concentration of the detergent/solvent decreases the cationic lipid self-assembles with the pDNA into lipoplexes.

The detergent dialysis method was initially used for preparing relatively stable cationic lipid/DNA lipoplexes by dissolving pDNA and cationic lipid mixture in a detergent solution, which is followed by a successive dialysis process to remove the detergent. The resulting particles did not show higher *in-vitro* transfection activity than the mixed lipoplexes, however, they have shown potency to fully protect pDNA following incubation with *Escherichia coli* DNase I (Wheeler et al., 1999; Monck et al., 2000; Tam et al., 2000; Judge et al., 2006). However, so far the physicochemical characteristics of these non-viral carriers and the factors which influence the preparation by detergent octylglucoside dialysis are rarely addressed.

7. Conclusive Summery

The present study describes the preparation and characterisation of gene carriers prepared by detergent N-octyl-beta-D-glucopyranosid (OGP) dialysis technique compared to mixed carriers. For the evaluation of these non-viral carriers we performed different approaches:

1. Study of physicochemical characteristics of lipid-based/pDNA complexes prepared by detergent OGP dialysis in terms of hydrodynamic diameter, zeta potential values compared to lipoplexes prepared by mixing technique **(Chapter 3)**.
2. In the second part of this chapter, we have estimated the condensation state of pDNA in dialysed lipoplexes by means of pDNA release using PicoGreen® assay as a marker for nucleic acid. Different anionic liposomes were applied to initiate pDNA release from dialysed lipoplexes compared to lipoplexes prepared by mixing technique **(Chapter 3)**. The effect of acidic pH on the pDNA release from different lipoplexes by anionic lipids has been also estimated.
3. The effect of detergent OGP dialysis on the morphological features of the dialysed DOTAP/pDNA lipoplexes was visualized using cryo-TEM. Mechanisms involved in the pDNA release from DOTAP/pDNA lipoplexes by DOPS/DOPE/DOPC (1:1:2) and OA/DOPE/DOPC (1:1:2) liposomes were visualized **(Chapter 4)**.
4. Lipid mixing estimation techniques were used to assess the disassembly of the lipoplexes by different negatively charged liposomes **(Chapter 5)**.
5. The detergent OGP dialysis technique was also utilized for preparation of polymer based-pDNA complexes. Polyplexes consisting of branched 25 kDa polyethylenimine polymer (PEI) and pDNA **(Chapter 6)** were characterized regarding their physicochemical as well as biological activities.

We exploit the principle of detergent dialysis approach to emphasize the importance of the structural properties of the pDNA complexes based on the physicochemical characteristics, the morphological features, and the differences compared to lipid mixing differences. The potential role of the non-ionic detergent OGP was discussed.

In **Chapter 3**, the OGP-based dialysis technique was used as an alternative method to prepare pDNA lipoplexes. Instead of direct mixing of cationic lipid and pDNA, dialysed lipoplexes were formed in the presence of non-ionic detergent OGP followed by dialysis to substitute the detergent. Two types of cationic lipid were used to prepare the lipoplexes: DOTAP, a quaternary amine containing lipid and DC-cholesterol, a tertiary amine containing lipid. The cationic lipid and pDNA were mixed in two different N/P ratios of 4 and 5 based on previous reports.

Laser light scattering technique revealed that the preparation technique influenced the hydrodynamic diameter and the zeta potentials of the lipoplexes. For dialysed DOTAP/pDNA lipoplexes, the

hydrodynamic diameter was characterised by larger sizes presumably due to a tendency of aggregation compared to mixed lipoplexes independent from the N/P ratio. For DC-cholesterol/pDNA lipoplexes the size was not dependent on preparation technique and the N/P mixing ratios. The structural differences between amine head groups of DOTAP (quaternary amine group) and DC-cholesterol (tertiary amine group) may play a role in the assembly of lipoplexes. Nevertheless, the mean size of dialysed lipoplexes exceeded by far the size limit for successful endocytosis in most cells.

Concomitantly with the size increase, the detergent dialysis technique produced lipoplexes showing a reduction in the zeta potential compared to mixed lipoplexes. This was observed for both DOTAP and DC-cholesterol based lipoplexes. Zeta potentials have a potential impact in stabilizing the colloidal properties of the lipid systems. In addition, it is well known that the endocytic uptake of lipoplexes into cells is based on the interaction of the positive charge of lipoplexes and the negatively charged cell membrane (Liang and Chou, 2009).

Additionally, the effect of detergent dialysis technique on pDNA compaction in the lipoplexes was investigated. Disassembly of the lipoplexes must occur before mRNA transcription can proceed. According to that concept, pDNA release should be added to the list of barriers to non-viral gene delivery. DOPS, a major anionic component of endosomal membrane, and oleic acid, a minor cellular lipid, were used to initiate pDNA de-condensation from lipoplexes. Of all lipoplexes tested, only dialysed lipoplexes showed always considerably neglected pDNA release. The pDNA release was also dependent on the mixing N/P ratios used to prepare the lipoplexes.

DOPS with different phospholipid compositions showed variable effect on the pDNA release from DOTAP/pDNA lipoplexes at N/P 4 and 5 ratios. The release of pDNA from mixed DOTAP/pDNA lipoplexes at N/P 5 was higher than N/P 4 when DOPS/DOPC liposomes were used. In contrast, the presence of DOPE showed a remarkable pDNA release from N/P 4 than at N/P 5.

The pDNA de-condensation in terms of the pDNA release rate from mixed DOTAP/pDNA lipoplexes by OA/DOPE/DOPC liposomes was higher than that observed by DOPS/DOPE/DOPC liposomes. Similar release pattern of dialysed DC-cholesterol/pDNA lipoplexes was also observed.

In order for nucleic acids to be delivered intact to cytoplasm or the nucleus of the cell there should be an escape mechanism from endo-/lysosomal pathway which is characterised by an acidic pH value. Therefore, the effect of acidic conditions on the pDNA release from lipoplexes was evaluated at pH 5.3. Mixed DOTAP/pDNA lipoplexes showed lower stability in acidic pH when DOPS containing liposomes were applied than that observed for DC-cholesterol/pDNA lipoplexes. A high increase in pDNA release compared to pH 7.4 was measured. This effect was not observed for dialysed lipoplexes. They exhibited similar pDNA release characteristics as observed at neutral pH.

From the obtained results, it can be concluded that the detergent induces cooperative interaction between cationic lipid and pDNA through hydrophobic interactions rather than only electrostatic forces as previously described for the mixing technique. This leads to lipoplexes characterized by lower zeta potential values and hence a higher tendency to aggregate. The pDNA compaction in the dialysed lipoplexes is considerably high.

Chapter 4 illustrated the influence of detergent OGP on the morphological characteristics of DOTAP/pDNA lipoplexes. In addition, the lipid organisation of DOTAP with negatively charged liposomes formed during pDNA release was visualized by cryo-TEM.

As also observed in PCS measurements the presence of non-ionic detergent OGP during cationic lipid/pDNA assembly produces lipoplexes shown by large aggregates in cryo-TEM. Both mixed and dialysed DOTAP/pDNA lipoplexes were arranged into lamellar forms. Dialysed DOTAP/pDNA lipoplexes showed dissimilar features than that visualized in case of mixed DOTAP/pDNA lipoplexes.

As previously shown for mixed DOTAP/pDNA lipoplexes, typical “spike” like aggregates were visualised. In contrast, the detergent dialysis method resulted in compact large structures with high electron dense particles indicating pDNA compaction. The interaction between pDNA and DOTAP cationic lipid showed similar features to “spaghetti” like aggregates. This was not described for DOTAP/pDNA lipoplexes before. The size of dialysed DOTAP/pDNA lipoplexes measured by cyro-TEM correlated well to size measured by PCS. Cyro-TEM confirmed the heterogeneity of both systems. A better understanding of the molecular assembly of pDNA and cationic lipids should help to establish correlations with their biological activity. Indeed, the transfection efficiency of a given lipid-DNA complex highly depends on its structural and physicochemical properties as well (Chesnoy and Huang, 2000).

In another part of the study, after interaction of DOTAP/pDNA lipoplexes with DOPS/DOPE/DOPC liposomes pDNA was observed to be released by lamellar-lamellar contact rather than non-bilayer fusion intermediate formation. The presence of lamellar phase lipid organisation for both mixed and dialysed DOTAP/pDNA lipoplexes was characterised by cryo-TEM. After longer contact, lamellar lipoplexes and DOPS/DOPE/DOPC liposomes were progressively disintegrated in case of mixed lipoplexes. Mixed lipoplexes retained their lamellar arrangement up to complete disintegration by DOPS/DOPE/DOPC liposomes. Although dialysed lipoplexes showed similar mechanism of pDNA release, they did not show a marked disassembly by anionic lipid DOPS after longer incubation.

A different mechanism of pDNA release from DOTAP/pDNA lipoplexes by oleic acid was visualized. OA/DOPE/DOPC liposomes formed an “inverted” non-bilayer phase transition with DOTAP lipid during pDNA release from DOTAP/pDNA lipoplexes. Although mixed and dialysed DOTAP/pDNA lipoplexes showed an “inverted” non-lamellar lipid phase separation, nevertheless, the size of the fusion

intermediates was more pronounced in mixed DOTAP/pDNA lipoplexes. This was indicated upon longer incubation time by an increase in size of fusion intermediates only in case of mixed DOTAP/pDNA lipoplexes.

According the previous findings, lipoplexes with an excess of cationic lipid rapidly released their pDNA content upon addition of anionic lipid liposomes. In the case of the lipoplexes with excess pDNA, the release of pDNA was quite inefficient even after 1 h of incubation with the same anionic lipids (Koynova and MacDonald, 2007).

We concluded that the presence of detergent during pDNA and cationic lipid assembly enhances the pDNA charge neutralization by the cationic lipids. After detergent dialysis, a considerable amount of cationic lipids is involved in the lipoplexes formation a condition where a condensation effect on pDNA might occur. This was clearly observed with the enlargement of fusion intermediate size during pDNA release which depends on the presence of free cationic lipids. In accordance, this was observed by a marked reduction in zeta potential and hence higher tendency for aggregation. These observations were accompanied by higher pDNA compaction in the lipoplexes and lower pDNA release. Additionally, the extent of pDNA release from DOTAP based lipoplexes by anionic lipids does not universally correlate with the propensity of the cationic/anionic lipid mixtures to adopt non-bilayer motifs. Only the rate of pDNA release could be enhanced.

Next, **in chapter 5**, the stability by means of dissociation and disassembly of the DOTAP/pDNA and DC-cholesterol/pDNA lipoplexes after incubation with DOPS and oleic acid lipids has been investigated. To assess lipoplexes disassembly, probe dilution assays of octadecyl-rhodamine OD (R18) and NBD-PE using fluorescence resonance energy transfer (FRET) were used.

The lipoplexes disassembly was characterised by lipid mixing between lipoplexes and different negatively charged liposomes using OD (R18). However, the extent of lipid mixing of dialysed DOTAP/pDNA lipoplexes with DOPS/DOPE/DOPC liposomes was not correlated with their pDNA release shown in **chapter 3**. This lack of correlation has been attributed to the fact that apparent lipid mixing of dialysed DOTAP/pDNA lipoplexes with DOPS containing liposomes induced presumably by fluorophore dilution only in the outer-monolayers of the contacted membranes. As concluded, the lipid mixing is not necessarily resulted in the concomitant release of pDNA from the dialysed DOTAP/pDNA lipoplexes, a prerequisite for pDNA to become eventually transcriptionally active. A similar result was in line with FRET result for the same lipoplexes. The energy transfer efficiency (*E*) indicated that mixed and dialysed DOTAP/pDNA lipoplexes showed a massive aggregation with DOPS/DOPE/DOPC liposomes. This was indicated by complete energy transfer efficiency loss. OA/DOPE/DOPC liposomes showed similar effects as observed with DOPS containing liposomes with same phospholipids combination. Both techniques

showed that mixed DOTAP/pDNA lipoplexes were loosely bound to pDNA accompanied by concomitant pDNA release.

The lipid mixing assays described in this chapter revealed that pDNA release from dialysed DC-cholesterol/pDNA lipoplexes was correlated with pDNA release observed in **chapter 3.** Moreover, the FRET assay revealed higher stability of mixed DC-cholesterol/pDNA lipoplexes as well as dialysed lipoplexes when mixed with DOPS or oleic acid liposomes. This was indicated by initial high energy transfer efficiencies.

Chapter 6 describes the use of the detergent dialysis approach to prepare polyplexes composed of pDNA and 25 kD polyethylenimine (PEI). Polyplexes were formed by an alternative technique complexing pDNA and PEI in the presence of the non-ionic detergent N-octyl-beta-D-glucopyranosid (OGP) at different N/P ratios followed by dialysis. Dynamic laser light scattering technique was applied to determine the influence of OGP on the hydrodynamic diameter and the zeta potential values of the polyplexes. Using the PicoGreen® assay, as a nucleic acid dye, the pDNA condensation state was estimated at alkaline pH values. By means of biological activity, gene expression efficiency of the polyplexes was characterized in kidney cell line (HEK 293) using flow cytometric analysis.

We showed that PEI/pDNA polyplexes can be formulated in the presence of the detergent OGP to form condensed pDNA. Unlike regular polyplexes, dialysed polyplexes exhibited different physicochemical properties as well as biological activities. As previously observed for lipoplexes, the preparation technique influenced the hydrodynamic diameter, zeta potential as well as release of pDNA from the polyplexes. In addition, the effect was OGP (50-200 mM) concentration dependent.

The hydrodynamic diameters of polyplexes at N/P 5 and 10 prepared by simple mixing were 111 and 161.7 nm, respectively. Zeta potentials of both complexes ranged between 30 and 35 mV. In contrast, at the same N/P ratios all dialysed polyplexes showed reduced zeta potentials compared to the mixed complexes. The zeta potential further decreased with increasing concentration of OGP in the range 50-200 mM used for polyplex preparation. This effect was more pronounced for polyplexes prepared at N/P 5 than at N/P 10. The reduced zeta potentials correlated with an increase in size and a tendency of the dialysed polyplexes to aggregate. Plasmid DNA was condensed in mixed as well as in dialysed polyplexes at N/P ratios 5 and 10. However, at pH 11 mixed polyplexes released approximately 80% of the incorporated pDNA independent on N/P ratio. In contrast, all dialysed polyplexes formed very stable systems and showed a lower pDNA release (maximum: about 30%) compared to the mixed polyplexes with a reduction of release with increasing N/P ratios. Mixed polyplexes showed GFP expression up to 47 % of cells with increasing amount of pDNA and a decrease in N/P ratio of polyplexes. At N/P ratios higher than 10, cytotoxic effects of the polyplexes were detectable. In comparison, transfection efficacies

of dialysed polyplexes were lower than for the mixed polyplexes with < 10% of all cells being positive for GFP. An increase in GFP expression was detectable with increasing amounts of pDNA and prolonged incubation time.

7.2. References

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Appendix

I. Acknowledgements
II. Curriculum Vitae
III. Conference Contribution and Publications
IV. List of Abbreviations

I. Acknowledgements

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I. Acknowlegments

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Sincerely,

Gihan Mahmoud

II. Curriculum Vitae

Personal Data

Surname:	Mahmoud
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Academic Background

1. Faculty of Pharmacy, Department of Pharmaceutical Technology, Friedrich Schiller University of Jena, Germany

Degree: Doctor of Philosophy study July 2005-present

Title: Innovative Vectors for Non-viral Gene Therapy using Detergent Dialysis Technique

2. Faculty of Pharmacy, Department of Pharmaceutical Technology, Helwan University/ Cairo,Egypt

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3. Faculty of Pharmacy, Cairo University,Cairo,Egypt

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Professional Background

1. Assistant Lecturer

Faculty of Pharmacy, Department of Pharmaceutical Technology, Helwan University, Cairo, Egypt

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Senior instructor, senior demonstrator as well as tutor of the laboratory works for undergraduate students, concerning formulation of pharmaceutical dosage forms, pharmacokinetics calculations, pharmacy practice and pharmaceutical calculations.

2. Teaching Assistant

Faculty of Pharmacy, Department of Pharmaceutical Technology, Helwan University, Cairo, Egypt

January 1997-2002

Instructor, demonstrator and tutor for above described subjects.

3. Assistant Researcher

National Organization of Drug Control and Research (NODCAR), Cairo, Egypt

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Registrar for commercial pharmaceutical products as well as commercial cosmetic products

Courses

German language intensive course: Mittelstufenpruefung (ZMP) Certificate GOETHE Institute, Cairo, Egypt

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International Computer Driving License (ICDL), a Certificate for Basic Computer Skills awarded by UNESCO Office, Cairo, Egypt

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II. Curriculum Vitae

Professional Activities

Teaching medical cosmetic courses to graduate students, Faculty of pharmacy, Helwan University, Cairo, Egypt

2001

Awards/ Recognition For Academic Excellence Received

1. Disposition and Targeting of Glutathione in Rabbit Eye Lenses from Liposomal Collagen Corneal Shields

Ministry of State for Environmental Affairs, International Ozone Day
Cairo, Egypt 16th September 2002

2. The Scientific Research Council-Award of Helwan University.
The academic outstanding degree to the master thesis for the academic year 2001/2002.

Research Experiences

- Preparation Techniques of Liposomes
- Physicochemical Characterization of Liposomes using Photon Correlation Spectroscopy (PCS).
- Plasmid DNA Extraction and Purification From Escherichia Coli Bacterial Cell Culture
- Preparation of Plasmid DNA Non-Viral Based Transfection Complexes
- Cell Culture Techniques
- In-Vitro pDNA Transfection Protocols Using Encoded Genes (ex. GFP)
- Cellular Uptake Protocols of Liposomes using Fluorescent Probes.
- Flow Cytometry Analysis Techniques
- Monitoring Drug Release Based on Spectroscopic Techniques
- High Performance Liquid Chromatography (HPLC) Analysis Techniques.
- UV/Vis Spectroscopy

Language Proficiency

Arabic Language: Native

English Language: Excellent (international TOEFL)

German Language: Fluent (ZMP)

III. Publications and Conference Contributions

Publications:

1. Gihan Mahmoud, Alfred Fahr and Dagmar Fischer

Characterization of pDNA/Polyethylenimine Polyplexes Prepared by Detergent Dialysis

" In preparation"

2. Gihan Mahmoud, Alfred Fahr and Dagmar Fischer

Cryo-TEM and Disassembly Studies of Non-Viral Lipid-Based pDNA Particles Prepared by Detergent Dialysis

"In preparation"

Oral Presentation:

1. DNA Cationic Lipid Complex: Characterization and Disassembly Studies
   Gihan Mahmoud and Alfred Fahr
   Liposome Workshop Seminar in Meiningen, 8th -10th November 2006
2. Proton transfer: An Approach to Assess DNA Dissociation from Differently Prepared Lipoplexes
   Gihan Mahmoud and Alfred Fahr
   Controlled Release Society German (CRS) Chapter Annual Meeting, 4th /5th March 2008; Technical University of Braunschweig

Poster Presentation:

1. Polyplexes of Plasmid DNA and Polyethylenimine: Preparation by Detergent Dialysis Technique
   Gihan Mahmoud, Alfred Fahr and Dagmar Fischer
   Controlled Release Society (CRS) German Chapter Annual Meeting, 19th /20th March 2009; Martin-Luther University of Halle-Wittenberg
2. Characterization of pDNA/Polyethylenimine Polyplexes Prepared by Detergent Dialysis
   Gihan Mahmoud, Alfred Fahr and Dagmar Fischer
   Controlled Release Society (CRS) 36th Annual Meeting and Exposition, 18th - 22nd July 2009 at the Bella Center, Copenhagen, Denmark

IV. List of Abbreviations

ADA	Adenosine Deaminase
AIDS	Acquired Immune Deficiency Syndrome
BPEI	Branched Polyethylenimine
CMC	Critical Micelles Concentration
Cyro-TEM	Cryo Transmission Electron Microscope
DC-cholesterol	Cholesteryl 3β-N-(dimethylaminoethyl) carbamate hydrochloride
DDAB	Dimethyl dioctadecylammonium bromide
DODAC	Dioleoyldimethylammonium chloride
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
EDOPC	*o*-ethyl-dioleoylphosphatidylcholinium
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOPG	1,2-dioleoyl-sn-glycerophosphate
DOPS	1,2 dioleoyl-sn-glycero-3-phospho-l-serine
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DOTMA	N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride
EGFP	Enhanced Green Fluorescence Protein
FRET	Fluorescence Resonance Energy Transfer
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H_{II}	Inverted Hexagonal Phase
kDa	Kilo-Dalton
$L\alpha$	Lamellar Phase
MMs	Mixed Micelles

IV. List of Abbreviations

MVs	Mixed Vesicles
MW	Molecular Weight
N/P ratio	Nitrogen to Phosphate ratio
NaCl	Sodium Chloride
NBD-PE	N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phosphatidylethanolamine
NLS	Nuclear Localizing Signal Peptide
OA	Oleic acid
OD (R18)	Octadecyl-rhodamine OD (R18)
ODNs	Oligodeoxynucleotides
OGP	N-octyl β-D-glucopyranoside
ONs	antisense phosphodiester oligonucleotides
PAMAM	polyamidoamine
PDI	Polydispersity Index
pDNA	Plasmid Deoxyribonucleic Acid
PEG	polyethylene glycol
PEG-CerC20	1-O-(2'-(ω-methoxypolyethyleneglycol) succinoyl)-2-N arachidoylsphingosine
POPC	palmitoyl oleoyl phosphatidylcholine
Rhd-PE	N-1,2-dioleoyl-sn-Glycero-3 Phosphoethanolamine-N- (lissamine Rhodamine B sulfonyl)

IV. List of Abbreviations

RNA	Ribonucleic Acid
SAXD	Small Angel X-ray Diffraction
siRNA	small interfering RNA
SPLP	Stabilized Plasmid Lipid Particles
λ_{ex}	excitation wavelength
λ_{em}	emission wavelength
PCI	Photochemical Internalization
CPP	Cell Penetrating Peptides
SCID	Sever Combined Immuno-Defficiency

Selbständigkeitserklärung (Own Work Declaration)

Hiermit erkläre ich,

dass die von mir vorgelegte Arbeit durch meine Person selbständig und nur unter Verwendung der aufgeführten Hilfsmittel und Literatur angefertigt wurde.
Ich habe weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch unmittelbar oder mittelbar geldwerte Leistungen in Zusammenhang mit dem Inhalt meiner Dissertation an Dritte erbracht.
Die vorliegende Dissertation habe ich ausschließlich an der Friedrich-Schiller Universität Jena als Prüfungsarbeit eingereicht wo mir die Promotionsordnung der Biologisch-Pharmazeutischen Fakultät bekannt ist.

Jena, im Juni 2009